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Novel polymeric systems based on natural materials: development and biological performance

Dissertação submetida à Universidade do Minho para obtenção do grau de Mestre em Processamento e Caracterização de Materiais

Orientador:
Prof. Doutor Rui Luis Gonçalves dos Reis

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ABSTRACT

Tissue Engineering has been widely studied as an alternative approach to treat tissue defects resulting from a disease or injury. Most of these studies use materials developed into different forms/structures as templates for cell attachment (or entrapment) and proliferation.

The present thesis, focused on the development and characterization of distinct natural origin polymeric systems that can find different applications in tissue engineering.

The first system studied consists of membranes and 3D-scaffolds based on chitosan, a biodegradable polymer of natural origin that shows adequate properties for several potential biomedical applications such as wound healing, skin substitutes, drug delivery, cell encapsulation and others. In this work, it was evaluated the general cytotoxicity and biocompatibility of several different chitosan membranes and 3D-chitosan scaffolds formulations, but the major focus was on the assessment of the effect of plasma surface treatments on the ability of chitosan membranes to promote cell adhesion and proliferation. For this purpose, the surface of the chitosan based membranes was modified by plasma treatment, using argon and nitrogen gas. Different *in vitro* cell culturing assays performed (MEM, MTT, MTS) showed that such treatments were useful to obtain enhanced cell attachment and proliferation on chitosan membranes. In fact, chitosan membranes treated by plasma, demonstrated a high potential for biomedical applications, such as substrates for skin tissue engineering. Additionally, plasma treatment procedure seems to have potential to be implemented in other chitosan-based materials, such as scaffolds for tissue engineering.

The second part of the work developed under this thesis was focused on the development of a novel cell encapsulation system based on two different natural origin polymers, namely alginate, a well know polymer widely studied for cell encapsulation, drug delivery, and carrageenan, a natural origin polymer that has not been significantly studied for biomedical applications despite its intrinsic interesting properties. Cell encapsulation systems allow to obtain constructs with a homogenous

cell distribution that can adapt to the shape of the tissue defect to be treated, as the microcapsules containing the cells can be easily injected into a tissue defect, using non-invasive surgical procedures.

Microcapsules were developed using *kappa* and *iota*-carrageenan, as well as a mixture of sodium alginate and *iota*-carrageenan. In all cases, chitosan was used to form the membrane of the capsules. All the developed capsules exhibited spherical shape, smooth surface and revealed good stability in PBS and in culture medium. The obtained results demonstrate that the incorporation of *iota*-carrageenan improves the stability of alginate based capsules and suggest that these capsules show adequate permeability for cell encapsulation without the need for additional citrate treatments (as it happens for alginate capsules). In fact, cell encapsulation experiments, using sodium alginate and *iota*-carrageenan (30/70%) capsules demonstrated that is possible to maintain cellular viability, demonstrating the potential of the developed system for tissue engineering applications.

RESUMO

A engenharia de tecidos tem sido estudada extensamente como uma alternativa para o tratamento de tecidos danificados por uma doença ou trauma. A maioria destes estudos utiliza materiais desenvolvidos para o efeito com formas/estruturas diferentes, como suportes para a proliferação e adesão de células (ou encapsulamento de células em sistemas poliméricos).

O trabalho apresentado, focou-se no desenvolvimento e caracterização de sistemas poliméricos de origem natural distintas com aplicações diferentes em engenharia de tecidos.

Na primeira parte do trabalho estudou-se à biocompatibilidade de membranas e estruturas porosas ("*3D-scaffolds*") baseadas no quitosano, um polímero biodegradável de origem natural, que exibe propriedades adequadas para diversas potenciais aplicações biomédicas, relacionadas com a cicatrização de feridas, suportes para engenharia de tecidos, libertação controlada de fármacos e encapsulamento de células, entre outras. Foi avaliada a citotoxicidade e a biocompatibilidade de membranas com diferentes formulações e estruturas tridimensionais porosas baseadas no quitosano, sendo o objectivo principal a avaliação do efeito do tratamento de plasma na superfície das membranas de quitosano na promoção a adesão e proliferação de células. A superfície das membranas de quitosano foi modificada utilizando um tratamento por plasma, usando árgon e azoto. Foram realizados diferentes testes de culturas celulares *in vitro* (MEM, MTT, MTS), os quais demonstraram que os tratamentos por plasma, melhoram significativamnte á adesão e proliferação de células nas membranas de quitosano. De facto, as membranas de quitosano tratadas por plasma demonstraram um elevado potencial para aplicações em engenharia de tecidos.

A segunda parte do trabalho consistiu no desenvolvimento de um novo sistema para encapsulamento de células baseado em dois polímeros diferentes, ambos de origem natural, nomeadamente o alginato, um conhecido polímero extensamente estudado para encapsulamento de células e em sistemas para a libertação de fármacos, e o carragenano que, apesar exibir algumas propriedades intrínsecas

interessantes, não tem sido muito estudado para aplicações biomédicas. Os sistemas de encapsulamento de células permitem obter sistemas híbridos tridimensionais de células e materiais (constructs) com uma distribuição celular homogénea que podem adaptar-se à forma do defeito do tecido a ser tratado, uma vez que as microcápsulas podem ser facilmente injectadas no defeito a tratar, utilizando técnicas cirurgicas de invasão mínia. Estes sistemas híbridos podem ser usados para a regeneração de diversos defeitos tecidulares e podem também ser usados simultaneamente como veículos para a libertação de agentes bioactivos.

As microcápsulas foram desenvolvidas usando *kappa*-carragenano e o *iota*-carragenano assim como misturas de alginato de sódio e de *iota*-carragenano. Em todos os casos foi utilizado o quitosano para formar a membrana envolvente das cápsulas. Todas as cápsulas desenvolvidas exibem uma forma esférica, uma superfície lisa e estabilidade adequada em PBS e no meio de cultura. Os resultados obtidos demonstraram que a incorporação do *iota*-carragenano melhora a estabilidade das cápsulas baseadas em alginato, o que indica que estas cápsulas apresentam permeabilidade adequada para o encapsulamento de células sem a necessidade de um tratamento adicional de citrato (como acontece para as cápsulas de alginato). Assim sendo, os ensaios de encapsulamento de células em cápsulas feitas de alginato de sódio e *iota*-carragenano (30/70%) provaram que é possível manter a viabilidade celular, demonstrando o potencial do sistema desenvolvido para aplicações em engenharia de tecidos.

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CHAPTER 1

1 INTRODUCTION

1.1 POLYMERS FOR BIOMEDICAL APPLICATIONS

In the past years, the field of Tissue Engineering has opened a new and exciting range of applications for biodegradable polymers.

Tissue Engineering is an emerging interdisciplinary field and one of the major components of regenerative medicine which follows the principles of cell transplantation, materials science, and engineering towards the development of biological substitutes that can restore and maintain normal tissue function [1].

Biomaterials, in general, provide a space (2D or 3D) for the cells to form into new tissue with appropriate structure and function. Moreover, the biomaterials may allow for the delivery of cells and appropriate bioactive factors, to the desired site in the body [1, 2]. As the majority of mammalian cell types are anchorage dependent, and will die if no cell adhesion substrate is available, the biomaterials play an important role as support structures for tissue regeneration. For this type of applications, the ideal biomaterial should be biodegradable and bioresorbable to support the replacement of normal tissue without inflammation [1, 2]. Degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate in order to keep the concentration of these degradation products in the tissue at a tolerable level [1, 3].

Although most of the research on Tissue Engineering has focused on polymers that have already been studied (and in some cases approved) for other clinical applications, the specific and highly demanding requirements for this type of use has encouraged the development of new and improved systems, both from natural and synthetic origin, as well as combination of natural with synthetic polymers.

Polymers are a promising class of biomaterials that can be engineered to meet specific end-use requirements. They can be selected according to key 'device' characteristics such as mechanical resistance, degradability, permeability, solubility

and transparency, but most of the currently available polymers need to be improved by altering their surface and bulk properties in order to make then suitable for biomedical applications. The design of macromolecules must therefore be carefully tailored in order to provide the combination of chemical, interfacial, mechanical and biological functions necessary for the manufacture of new and improved biomaterials (Figure 1-1) [4].

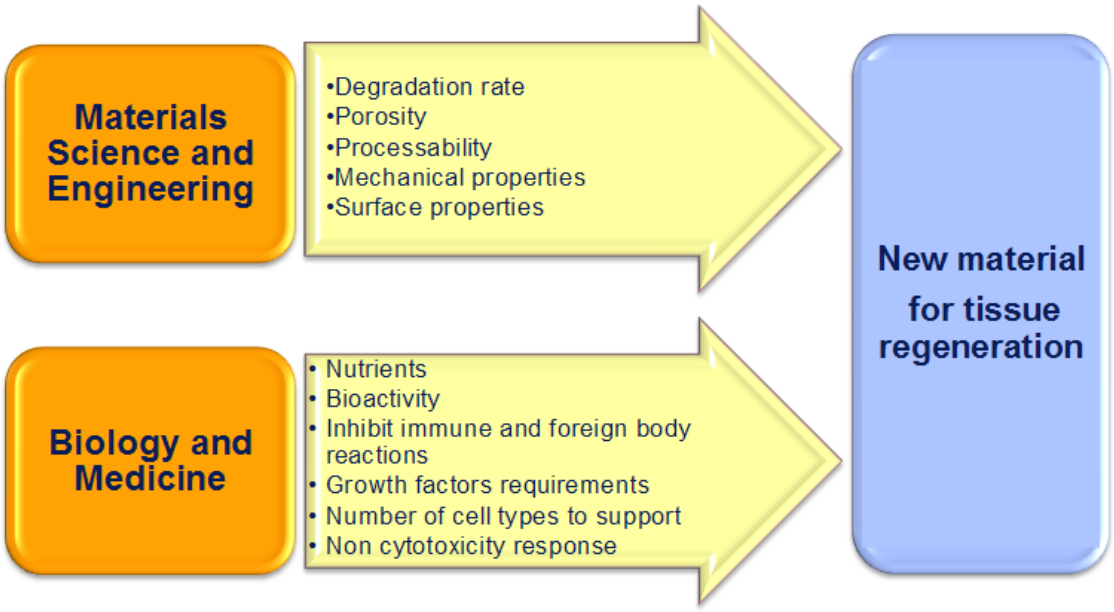

Figure 1-1. Factors to consider when designing a new biomaterial. Biological, medical and engineering properties must be integrated to achieve successful biomaterials for tissue regeneration. Adapted from [5].

Polymers have been used widely in medicine and biotechnology [4], in a wide range of applications such as surgical devices [6], implants [7], drug delivery systems , carriers of immobilized enzymes and cells [8], biosensors, bioadhesives, ocular devices [9] and materials for orthopaedic applications [10].

Natural origin polymers are abundant and usually biodegradable. Table 1-1, presents some of the most important natural and synthetic origin polymers and their main biomedical applications. The principal disadvantages of these polymers is related to the difficulty in the development of reproducible production methods, because their complex structure often renders modification and difficult purification [4]. In the case of synthetic polymers, these are available in a wide variety of compositions with readily adjusted properties. The main disadvantage of synthetic polymers is the

general lack of biocompatibility in the majority of cases, often associated with inflammatory reactions [4].

The work developed in this thesis has focused on the use of natural origin polymers such as chitosan, carrageenan and alginate and therefore, in the next sections, these materials will be further described.

Table 1-1. Polymeric biomaterials: main properties and applications. Adapted from [4]

Polymer	Main applications and comments	Ref
NATURAL POLYMERS		
Collagen	Absorbable sutures, wound dressing sponge, drug delivery microspheres.	[11], [12, 13]
Albumin	Cell and drug microencapsulation.	[14-18]
Poly(amino acids): poly(L- lysine), poly(L- glutamic).	Non toxic, non antigenic and biocompatible. Used as oligomeric drug carriers.	[19-21]
Carboxymethyl cellulose	Cell immobilization via a combination of ionotropic gelation and polyelectrolyte complex formation (e.g. with chitosan), drug-delivery systems and dialysis membranes.	[22-24]
Cellulose sulphate	Component of polyelectrolyte complex for immunoisolation of cells. Complex-forming ability is highly sensitive to degree of acetylation.	[25, 26]
Agarose	Largely used as supporting materials in clinical analysis and as an immobilization matrix.	[27, 28]
Alginate	Excellent gel-formation properties; its relative biocompatible; microstructure and viscosity are dependent on the chemical composition (batch to batch variations). Used as immobilization matrices for cells and enzymes, controlled release of bioactive substances, injectable microcapsules for treating neurodegenerative and hormone-deficiency disease.	[6, 29, 30]
Carrageenan	Excellent thermoreversible properties. Used for microencapsulation of microorganisms.	[31-33]
Hyaluronic acid	Excellent lubricant, potential therapeutic agent.	[11, 34]
Heparin and heparin-like glycosaminoglycanes	Antithrombotic and anticoagulant properties. Extensively used in surgery. Some are candidates for ionotropic gelation and capsule formation.	[35]
Dextran and its derivates	Excellent rheological properties. Plasma expander. Widely used as a drug carrier.	[36, 37]
Chitosan and its derivates	Biocompatible, non toxic, excellent gel and film forming ability, natural polycation. Widely used in controlled delivery systems (e.g. gels, membranes, microspheres).	[38-46]

Cont.

Polymer	Main applications and comments	Ref
SYNTHETIC POLYMERS		
Poly (lactic acid), poly (glycolic acid) and their copolymers	Used in sutures, drug delivery systems and in tissue engineering. Biodegradable. Often copolymerized to regulate degradation time.	[3, 47, 48]
Poly (hydroxyl butyrate), poly (caprolactone) and copolymers, poly (alkylene succinate), etc.	Biodegradable, used as a matrix for drug delivery systems, cell microencapsulation. Properties can be changed by chemical modification, copolymerization and blending.	[49]
Polyamides (nylons)	Sutures, dressing, haemofiltration and blending.	[50]
Polyethylene (low density)	Sutures, catheters, membranes, in surgery.	[51, 52]
Poly (vinyl alcohol)	Gels and blended membranes are used in drug delivery and cell immunoisolation.	[53]
Poly (ethylene oxide)	Highly biocompatible. Different polymer derivatives and copolymers have been utilized in a variety of biomedical applications.	[54]
Poly (hydroxyethyl methacrylate)	Hydrogels have been used as soft contact lenses, for drug delivery, as skin coatings and for immunoisolation membranes.	[9, 55]
Poly (methyl methacrylate)	This and its copolymers are used as dental implants and in bone replacement.	[56, 57]
Poly (tetrafluroethylene) (Teflon®)	Vascular grafts, clips and sutures, coating.	[58]
Polydimethylsiloxanes	A silicone. Implants in plastic surgery, orthopaedics, blood bags and pacemakers.	[59]
Poly (ortho esters)	Surface eroding polymers. Application in sustained drug delivery, ophthalmology.	[60-62]
Polyanhydrides	Biodegradable, useful in tissue engineering and for the release of the bioactive molecules.	[63, 64]

1.1.1 NATURAL ORIGIN POLYMERS FOR TISSUE ENGINEERING APPLICATIONS

Naturally derived materials have been used for tissue engineering because they offer several advantages related to biological recognition (being similar, often identical to macromolecular substances present in the ECM), by the biological environment, which is then prepared to recognize and to deal metabolically with these materials [4, 65].

1.1.1.1 Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit)

Figure 1-2), commercially produced by deacetylation of chitin [44, 66]. Chitin is a natural polymer found in many arthropods as shells of marine crustaceans (crab, shrimp and shellfish), insects and cell walls of fungi. This polysaccharide is the second most important compound in nature after cellulose. It is not toxic and is insoluble in water, as well in many other solvents such as acetic acid [39, 44, 67-69]. Due to the existence of primary amine groups, the structure can be protonated at low pH, and chitosan is found to be soluble in moderated acidic solutions.

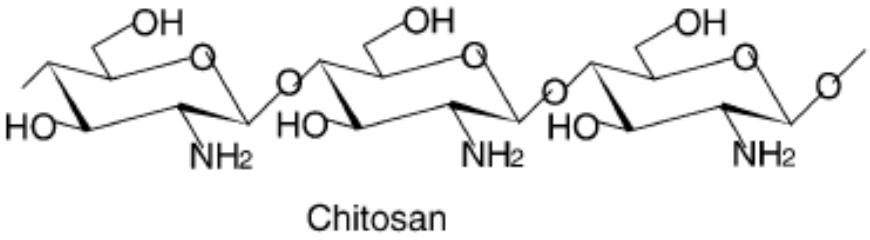

Figure 1-2. Chitosan structure, adapted from [70].

Chitosan can be formulated into a variety of forms including powders [71], gels [40], scaffolds [41, 72, 73], particles [41, 74], microspheres [75], microcapsules [29, 75, 76] and films [42, 77, 78] with an array of biological properties and applications (Figure 1-3) [39]. Furthermore, chitosan formulations allow a wide range of molecules to be attached. This property allows the delivery of growing factors that could help to optimize the physical and biological properties of this material as a matrix scaffold and vehicle system in tissue regeneration [39].

Chitosan was discovered in 1859, when Rouget boiled chitin in a concentrated potassium hydroxide solution, promoting the deacetylation of chitin [79]. Fundamental research on chitosan did not start until about a later century [29]. This natural polymer can selectively bind substances such as cholesterol, fats, metal ions, proteins and tumour cells. Others properties include antifungal effects, antibacterial [80], acceleration of wound healing [81, 82], stimulation of the immune system and acceleration of plant germination [29].

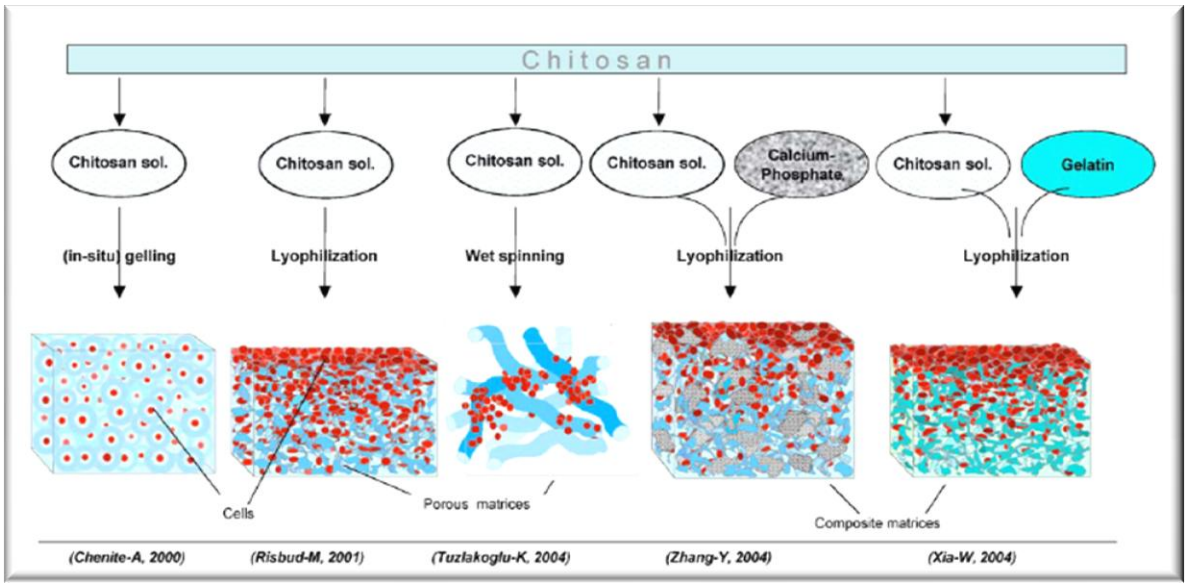

Figure 1-3. Examples of chitosan processing for uses in tissue engineering: Cells may be encapsulated in gels or seeded in porous matrices including sponge-like or fibrous structures. Combinations of chitosan with other biocompatible materials, such as calcium phosphate or gelatine, are applied to modify biomechanical and cell-matrix-interaction properties. Adapted from [68].

Several research works have shown that membranes formed from this polymer could be explored for surgical dressing, guided tissue regeneration and controlled release applications [1, 67].

Chitosan has been the best researched version of the biopolymer because of its ready solubility in dilute acids, rendering the material more accessible for utilization and chemical reactions [41]. Chitosan in aqueous acid solutions reacts with anionic polysaccharides such as: carboxymethylcellulose [83], xanthan [84, 85], alginate [86, 87], carrageenan [31, 66], gellan [88], oxychitin and oxypullulan [89-91], chondroitin and hyaluronan [92, 93], poly (galacturonic) [94], poly (α-L-glutamic acid) [95] as well as synthetic polyanions such as poly (acrylic acid) [96], to give origin to the formation of polyelectrolyte complexes [97].

The 3B's Research Group of the University of Minho has been studying chitosan for a wide range of biomedical applications, summarized on Table 1-2, that includes some studies based on chitosan and others polymers for tissue engineering applications. These materials have been developed into different forms such as membranes [98-101], 3D-scaffolds [38, 73, 74, 102] and fibres [69] aiming at different tissue engineering applications. One of the aims of this work was to assess the biological

response of chitosan based membranes and scaffolds developed for biomedical applications.

Table 1-2. Some studies developed by the 3B's Research Group on chitosan based materials.

Name article	Refs.
Multichannel mould processing of 3D structures from microporous coralline hydroxyapatite granules and chitosan support materials for guided tissue regeneration/engineering. 2003.	[105]
Starch-chitosan hydrogels prepared by reductive alkylation crosslinking. 2004	[106]
Production and Characterization of Chitosan Fibers and 3D Fiber Mesh Scaffolds for Tissue Engineering Applications. 2004.	[69]
Preparation and characterisation in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. 2004.	[99]
Influence of β-Radiation Sterilization in Properties of New Chitosan/Soybean Protein Isolate Membranes for Guided Bone Regeneration. 2004.	[98]
Hydroxyapatite Reinforced Chitosan and Polyester Blends for Biomedical Applications. 2005.	[38]
Physical properties and biocompatibility of chitosan/soy blended membranes. 2005.	[101]
Functional nanostructured chitosan/siloxane hybrids. 2005.	[107]
Properties of Melt Processed Chitosan and Aliphatic Polyester Blends. 2005.	[108]
Use of Chemically Modified Chitosan and other natural-origin polymers in tissue engineering and drug delivery In Biodegradable Systems in Tissue Engineering and Regenerative Medicine. 2005.	[109]
Chitosan particles agglomerated scaffolds for cartilage and osteochondral tissue engineering approaches with adipose tissue derived stem cells. 2005.	[74]
Study of the fosfosal controlled permeation through glutaraldehyde crosslinked chitosan membrane. 2006.	[100]
Physicochemical Characterization of Novel Chitosan-Soy Protein/TEOS Porous Hybrids for Tissue Engineering Applications. 2006.	[102]
Enzymatic degradation behaviour of starch conjugated phoshorylated chitosan. 2006.	[110]

1.1.1.2 Carrageenan

Carrageenan is a linear heteropolysaccharide with ester sulphate groups. It's main chain consists of alternating copolymers of 1,4-α and 1,3-β-D-galactopyranose and 3,6-anhydro-D-galactopyranose [103, 104] (Figure 1-4). Carrageenan can be found in them three major forms that are designated by means of the Greek letters *κ* (*kappa*), *i* (*iota*) and *λ* (*lambda*). The main structural difference among them is in the sulphate group degree of substitution (Figure 1-5).

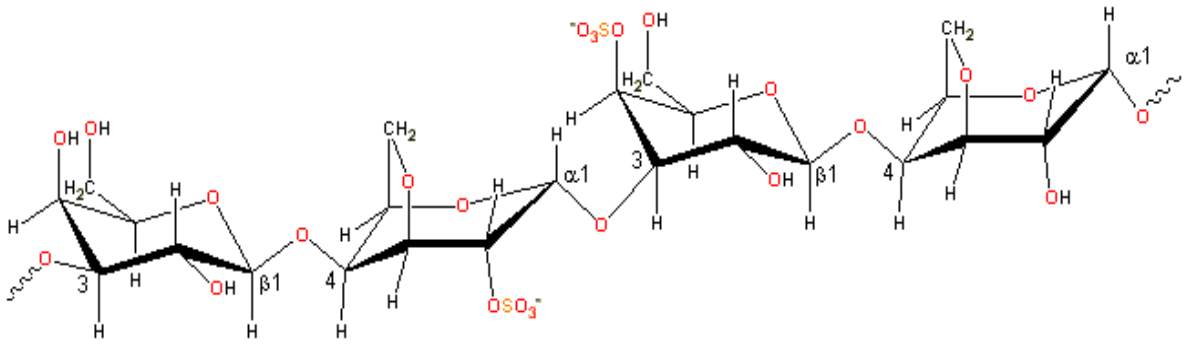

Figure 1-4. The main carrageenan chain consists of alternating 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units. Adapted from [115].

All carrageenans are highly flexible molecules which, at higher concentrations, organize into double-helical structures. Gel formation in *kappa*- and *iota*-carrageenans involves helix formation on cooling from a hot solution together with gel-inducing and gel-strengthening; the helix–helix aggregation relies upon specific cation presence (Ca^{2+} and K^{+}), which can screen electrostatic repulsive forces between the participating chains by packing within the aggregate structure [31, 111, 112].

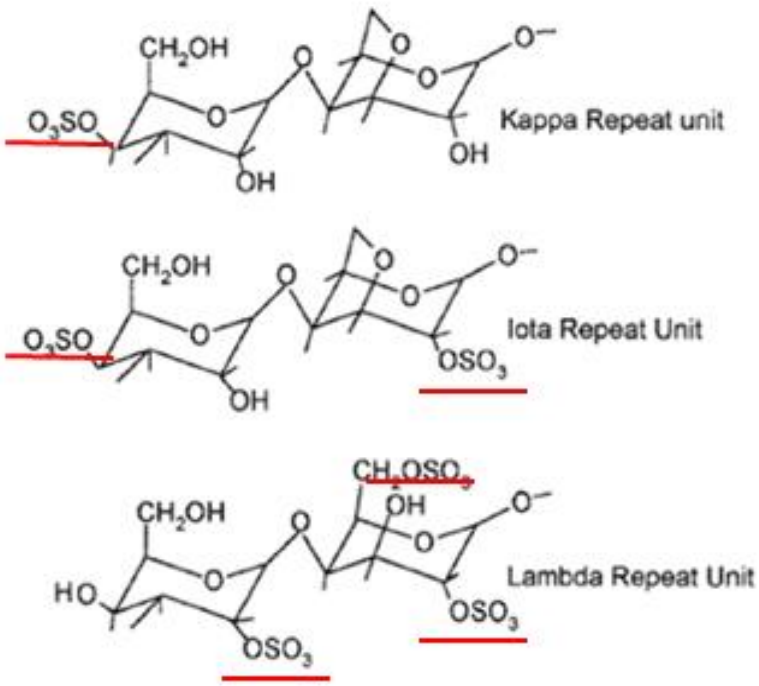

Figure 1-5. Chemical structures of *kappa*, *iota* and *lambda* carrageenans. Adapted from [117].

Kappa-carrageenan forms firm and strong rigid gels, while *iota*-carrageenan constitutes soft and elastic gels [31, 113]. *Lambda*-carrageenan does not gel as the high density of charged sulphate encourages an extensive conformation [114]. Table 1-3 presents some of the basic characteristics of different types of carrageenans.

Table 1-3. Main characteristics of the different forms of carrageenan. Adapted from [114].

Chemical composition	Hydrocolloid composed of α-D-1.3 and β-D-1.4 galactose residues that are sulphated at up to 40% of the total weight. Strong negative charge over normal pH range. Associated with ammonium, calcium, magnesium, potassium or sodium salts.
Solubility	*λ* is readily soluble in cold or hot aqueous solution. *K* is soluble in hot solution; treatment of aqueous solution with potassium ion precipitates.
Gel formation	*λ* does not form gels. *λ* and *i* form right-handed helices Potassium chloride promotes gel formation of *k*. Calcium ion promotes gel formation of *i*. Gel formation in *k* and *i* carrageenans involves helix formation on cooling from a hot solution together with gel-inducing and gel-strengthening K^+ or Ca^{2+} cations respectively
Metabolism	Hydrolisis of glycosidic linkages at lower pH, especially pH ≤3.0; also desulphation by sulphatases.
Source	Red algae; predominantly aqueous extraction from *Chondrus*, *Gigartina*, and various *Eucheuma* species.
Viscosity	Near logarithmic increase in viscosity with increasing concentration. Viscosity of food-grade carrageenan defined as not less than 5 cps at 75ºC for a 1.5% solution; viscosity ranges from 5 to 800 cps for 1.5% solution at 75ºC.
Molecular weight	Discrepancies in definitions. Native carrageenan reported to have average molecular weight of $1.5x10^6$ – $2x10^7$, food grade carrageenan reported as 100.000-800.000 or 200.000-400.000. Degraded carrageenan (poligeenan) has average molecular weight of 20.000-30.000.
Properties	*λ and k* combine easily with milk proteins to improve solubility and texture, serve as thickening agent, emulsifier, stabilizer.
Major uses	Milk products, processed meats, dietetic formulations, infant formula, toothpaste, cosmetics, skin preparations, pesticides, laxatives.
Concentration in food products	0.005-2.0% by weight.

Carrageenan has a structural role in the intercellular matrix material of numerous species of seaweeds of the class Rhodophyta, where its amorphous structure provides

the flexibility required to adapt to the varying stress of tidal and wave motion [103, 116]. The main substituent is the hemiester sulphate group. These strongly ionic groups mutually repel each other to maintain the molecule in a highly extended, flexible configuration [103].

Carrageenans are widely used in the food and other industries (cosmetic products, pesticides and pharmaceutical), as thickening and stabilizing agents. However, its predominant role has been in food preparations (a wide variety of food groups), because the carrageenans have the ability to substitute fat and to combine easily with milk proteins to increase solubility and improve texture [114].

Several investigations have associated carrageenan with the induction and promotion of intestinal neoplasm and ulceration in numerous animal experiments [118-121]. This behaviour is related with gastrointestinal metabolism of carrageenan, where food-grade carrageenan may be contaminated with low molecular weight carrageenan that may arise during food processing [114]. On the other hand, previous studies have demonstrated the benefits of carrageenans in health applications, and considered a useful microbicide that inhibits the human immunodeficiency virus (HIV-1) infection of epithelial at nanomolar concentrations. Moreover, carrageenans have also been considered as possible active agent against the cell-associated and cell-free virus in genital fluids [122]. The microbicide behaviour, was supported with recent studies where it has been demonstrated that carrageenans, as sulphated polysaccharides, are potent inhibitors of the infectivity of genital by human papilloma virus (HPV) and pseudovirus (PsV) *in vitro* [113, 123].

In the present work, carrageenan is proposed as an alternative polymer for the development of cell encapsulation systems. The use of carrageenans in the encapsulation field has rarely related in the literature. Previous studies have reported on capsules produced by carrageenan and oligochitosan polymer [31], but concerning cell encapsulation the reports are only related to the use of carrageenan (in special *k*-carrageenan) for the encapsulation of microbial cells [32, 33, 122].

1.1.1.3 Alginate

Alginate is a linear polymer of (1-4) - β -D-mannuronopyranosyl and (1-4)- α -L-guluronopyranosyl units in a copolymer that contains homopolymeric sequences

(figure 1-6). They occur naturally as the major structural polysaccharides of brown marine algae (Phaeophyceae) [124, 125] and as extracellular mucilages secreted by certain species of bacteria (*Azobacter Vinelandii*) [30, 124].

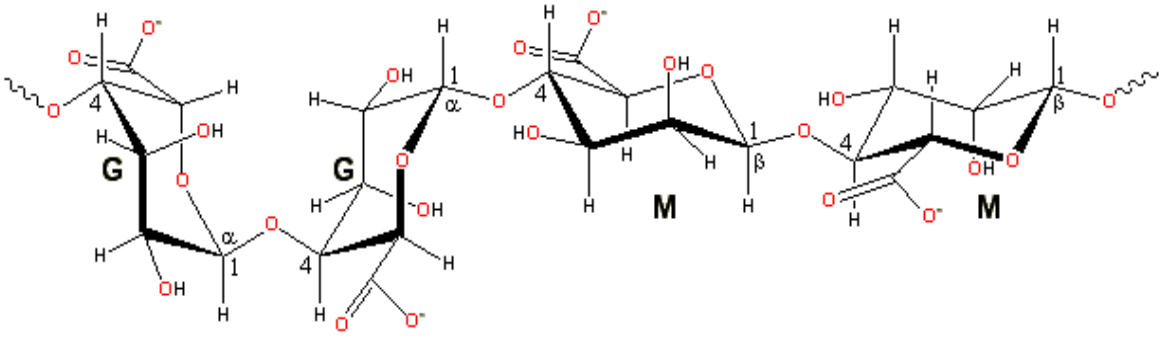

Figure 1-6. Alginate structure. Adapted from [143].

Guluronic and manuronic acid contain negatively charged functional groups. As a result, these negatively charged regions within the alginate structure can parallel the electrostatic conditions created by sulphated proteoglycans in native tissue and might offer to some cells, such as chondrocytes, a more conductive environment [126] .

Alginate remains the most widely used biomaterial for immobilizing cells, because of the many advantages it offers [127-129]. First, alginate has the capacity to gel under physiological conditions that are compatible with cell survival [128, 130]. The gelling reaction does not require toxic solvents and does not generate harmful by products. Second, the rapidity of the gelling process is a further critical advantage [128].

This polymer develops a simple and rapid gelation with divalent metal ions such as Ca^{2+}, through binding of consecutives blocks of G-molecules on individual or different molecules [124, 131-133]. Therefore, many researchers used alginate as the matrix to prepare microcapsules to be used as an injectable cell delivery vehicle [134] and cell immobilization matrix [135-137], involving different cell sources such as myoblasts [138, 139], epithelial cells [139] hybridoma cells [8, 140], fibroblasts [134, 137], pancreatic islets [6, 134] and microbial cells [141, 142]. Additionally, alginate is relatively biocompatible and approved by the Food and Drug Administration (FDA) for human use as wound dressing material [1].

As a natural polymer, alginate is limited by its tendency to be largely contaminated. Additionally, the industrial process used for extracting alginates from seaweed can introduce additional contaminants into the raw materials [128]. Consequently, the purity of the alginate has been found to be a pertinent factor in the biocompatibility of alginate [133]. For instance, when non purified alginate was used for islet encapsulation and transplantation into diabetic animals, a severe reaction against the microcapsule occurred immediately after implantation, and blood glucose was not normalized or was normalized only for a few days [128, 138]. On the other hand, alginate purification allowed the successful encapsulation and transplantation of islets, enabling the control of the levels of glucose in the blood of diabetic animals [128, 144].

The presence of immunologic response can be partly for linking to β(1-4) glycosidic linkages, since other homopolymeric di-equatorial polyuronates, like D-glucuronic acid (C6-oxidized cellose), exhibit this feature. *In vivo* animal models have revealed the immunologic potential of polymannuronate in diverse areas for protection against lethal bacterial infections and irradiation, and for increasing non-specific immunity [124].

Due to its interesting and promising properties, alginate was also studied in this thesis for the development of a novel encapsulation system. However, our aim was to combine this polymer with other (namely with carrageenan) in order to enhance the properties of this system as a cell encapsulation device, envisioning the future development of highly functional systems.

1.1.2 PROCESSING AND FORMAT OF NATURAL POLYMERS

Polymeric materials have been applied in many different biomedical applications, and therefore may be found in a variety of forms such as membranes, films, fibres, gels, hydrogels, capsules, spheres, particles and 3D-structures (scaffolds) (Figure 1-7).

In fact, in most cases, the criteria for the design of the polymeric systems depend on the target application/site of implantation [4]. Careful design of the structure and of the interfacial properties of the matrix and its morphology and pore size are

particularly important for biomaterials used as cell-growth scaffolds, and as supports for immobilized biomolecules/cells [4, 145].

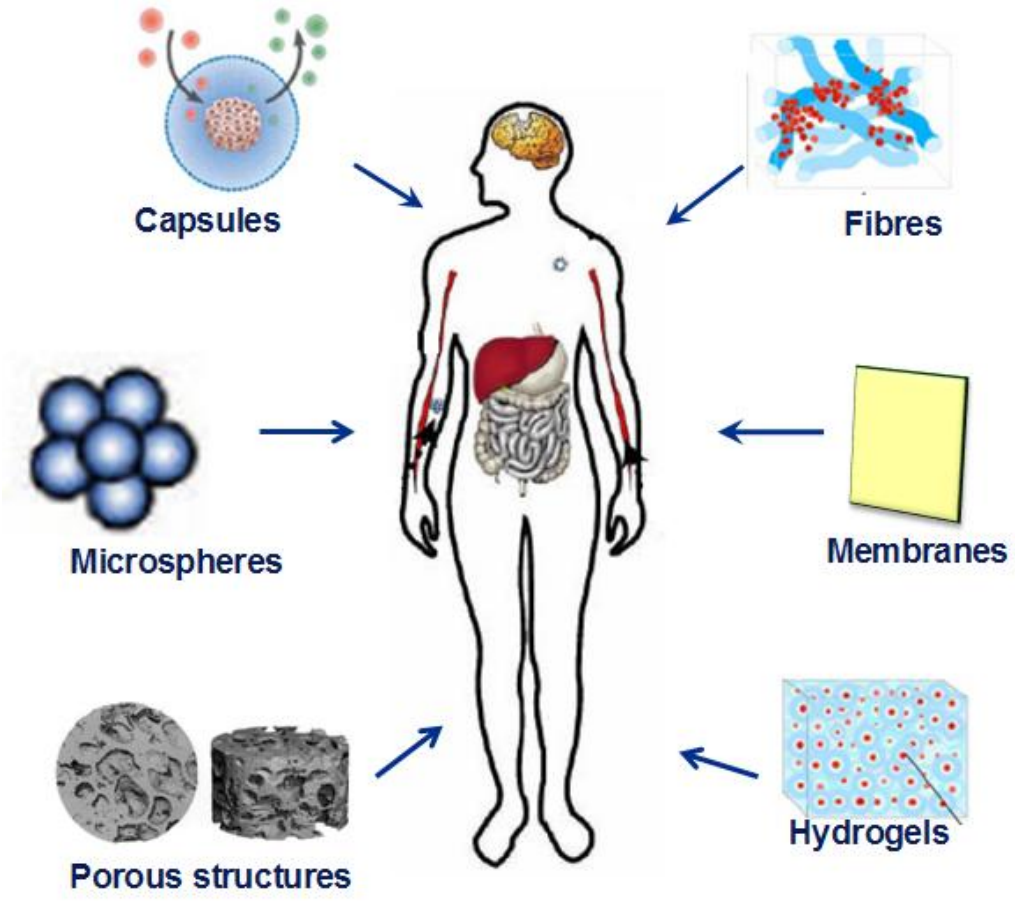

Figure 1-7. Some forms based on natural polymers that can be used in different biomedical applications, such as tissue regeneration and delivery systems.

Others requirements and properties such as durability, elasticity, tensile strength, adhesion should be considered in polymers for using as sutures and long-term skin substitutes. Polymers for matrices or carrieres in controlled-delivery devices require the consideration of characteristics like molecular weight, adhesion and solubility, depending on the type of system, action and the target site in the body. Polymeric materials for drug delivery must satisfy additional requirements such as environmental responsiveness (pH or temperature dependent phase or volume transformation) [4, 126].

In this work, we have focused on three particular designs/formats, based on different polymers (Figure 1-8), that may found use in particular tissue engineering applications or in other cell based therapies. For this reason, in the next sections we will develop the description of membranes, 3D-porous scaffolds and capsules, their main applications, features, and processing methodologies.

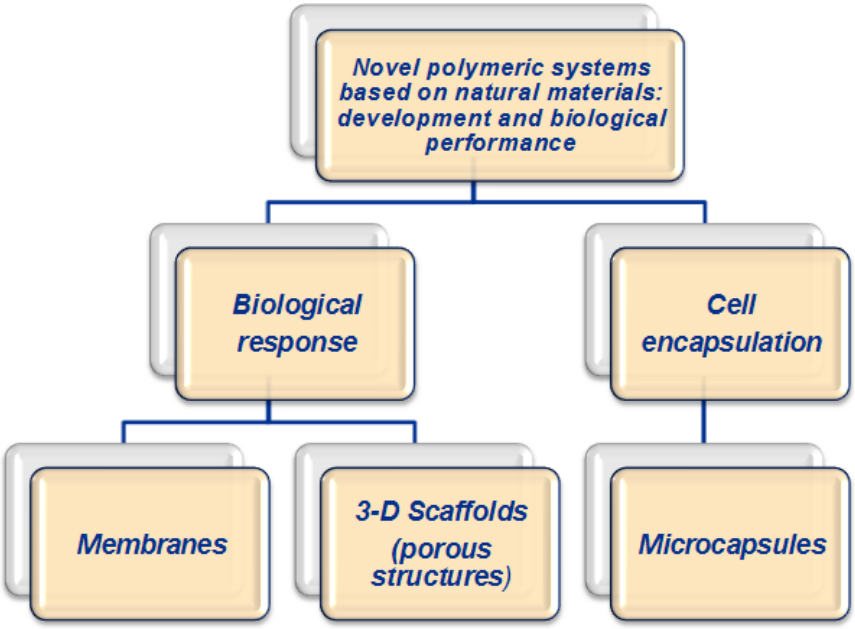

Figure 1-8. Schematic representation of the research approach followed in this thesis.

1.1.2.1 Membranes

Polymeric membranes have been developed for a variety of industrial applications such as microfiltration, ultrafiltration, reverse osmosis and gas separation [146, 147]. In biomedical applications, polymer membranes are used specially for skin regeneration, maxillofacial and pharmaceutical applications [126]. Each application needs to meet specific requirements regarding the material and structure [146]. The final morphology of the membranes obtained depends on the material properties and the processing conditions. The majority of membranes are prepared by methods based on a controlled phase separation of polymer solutions into two phases: one with a high polymer concentration and another with low polymer concentration [146]. Membrane systems prepared by this method take advantage of their selectivity, high surface-area-per-unit-volume, and their potential for controlling the level of contact and/or mixing between two phases [147].

Nevertheless, membranes can also be obtained by an interfacial polymerisation technique. In this case, the polymer is transformed in a controlled manner from a liquid to a solid phase. The concept of phase inversion involves a variety of different techniques such as precipitation by controlled solvent evaporation, thermal precipitation, precipitation from vapour phase, immersion precipitation, etc. [148].

Another method for preparing membranes is solvent casting, where the polymer is dissolved into a suitable organic solvent (e.g. chitosan can be dissolved into aqueous acetic acid) and then the solution is cast into a petri dish or into an appropriate mould [101] (Figure 1-9).

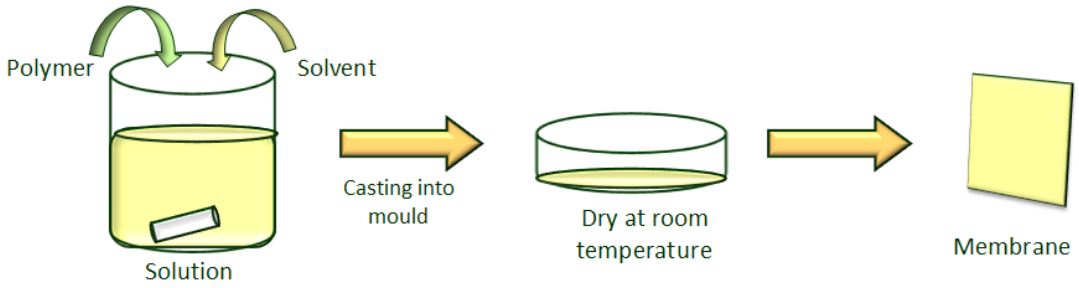

Figure 1-9. Membrane prepared by solvent casting – schematic representation.

Chitosan, for example is a polymer easily processed into membranes. In fact, chitosan membranes may be prepared by various ways such as evaporation of chitosan solvents, cross-linking with bifunctional reagents, chelating with anionic counterions or complexion with polymers and proteins [29]. Chitosan membranes have been used in several biomedical applications, such as controlled release [76, 149, 150], wound healing and surgical dressing [42, 151, 152]. In this work, solvent casting was the methodology used to obtain the chitosan based membranes.

1.1.2.2 Scaffolds design and fabrication

Three-dimensional (3-D) porous constructs have been one of the most widely studied scaffolds format in tissue engineering, as they provide the necessary support for cells to proliferate and maintain their differentiated function, and their architecture may define the ultimate shape of the new tissue to be formed, such as bone and cartilage [153, 154].

Factors governing scaffold design are complex and include considerations of matrix architecture, pore size and morphology, mechanics versus porosity, surface properties and degradation rate [153, 155].

A number of fabrication technologies have been applied to process biodegradable and bioresorbable materials into 3-D polymeric scaffolds of high porosity and surface area. The conventional techniques for scaffold fabrication include fibre bonding [156, 157], solvent casting [157-161], particulate leaching [162], gas foaming [163-165], phase separation/emulsification [166-169], membrane lamination and melt molding [153, 170]. Additionally, others methods include extrusion, *in situ* polymerization [73, 171], 3D-printing and selective laser sintering [155, 172, 173]. Below some methods for scaffolds fabrication that were used in this thesis are described.

Solvent-casting and particle leaching. The solvent casting and particle leaching is one of the most widely used methodologies to obtain scaffolds and membranes for biomedical applications. This method involves the use of a water soluble porogen, such as salt (NaCl) [159, 160, 174] to create pores. The solvent casting and particle leaching method consist in dispersing a sieved mineral (e.g. NaCl) or organic (e.g. saccharose) particles in a polymer solution [73]. This dispersion is then processed either by casting or by freeze-drying in order to produce porous bi and three-dimensional supports [175].

Freeze-Drying. The basic principle of the freeze-drying process relies on a thermally induced phase separation, which occurs when the temperature of a homogenous polymer solution, previously poured into a mould, is decreased. Once the phase-separated system is stabilized, the solvent-rich phase is removed by vacuum sublimation leaving behind the polymeric foam [175]. The foam morphology is of course controlled by any phase transition that occurs during the cooling step, i.e., liquid-liquid or solid-liquid demixing [175].

In this work, chitosan based porous structures were produced by means of combining a sol-gel process and the freeze-drying technique. This technique has been used in previous studies by our group for the development of chitosan based porous structures, where the chitosan sol-gel was poured into a petri dish and allowed to dry

at room temperature for several days [102]. By its turn, the porous hybrids were obtained by transferring the sol-gel into a mould, freezing at -80^{o}C overnight, followed by freeze-drying for several days to completely remove the solvent [102]. The properties, such as scaffold porosity, are controlled by several treatments including crosslinking, neutralization with alkali solutions and control of the pH of the initial polymeric solution [73].

1.1.2.3 Capsules

Cell encapsulation technology is an exciting field of biotechnology that has emerged and developed rapidly in the past decade [176]. One of its strategies is cell microencapsulation, which aims to overcome the present difficulties relating to whole organ graft rejection and, consequently, the requirements for the use of immunomodulatory protocols or immunosuppressive drugs [136]. Therefore, cell microencapsulation has also raised much interest in part due to the advancement and optimization of the biomaterials used to develop the capsules [177].

Capsules in the 0.3-1.5 mm range have been traditionally refereed to as microcapsules in the cell encapsulation field. Their relatively small size is considered advantageous from a mass transport perspective. Microcapsules are typically more durable than macrocapsules and difficult to mechanically disrupt. Numerous microencapsulation techniques, different in the nature of entrapment mechanism, have been developed. Besides traditional capsules with a well-defined shell-and-core structure, encapsulation in microbeads without a distinct membrane has been successful used in some specific applications [133].

Some known methodologies for capsules formation namely, complex coacervation, coextrusion and emulsion, polyelectrolyte complex and ionotropic gelation [129] are described below.

Coacervation: This methodology consists in using a system with some parameters such as temperature, pH, or composition, that can be adjusted in a liquid phase of pre-membrane component separates from a polymeric solution and wraps the liquid core as a uniform layer. The liquid core droplet is called “coacervate”. The pre-membrane component is solidified by means of heat, crosslinking, or solvent removal techniques. An hydrophilic pre-membrane component, such as gelatin and gelatin-gum acacia, is

required for the encapsulation of citrus oil, vegetable oil, and water-soluble vitamins. When the core material is water soluble or immiscible, the pre-membrane component is hydrophobic. Coacervation is an efficient but expensive process [141].

Emulsion/interfacial polymerization: In this method, interfacial polymerization occurs between monomers dissolved in the respective immiscible phases. Aqueous drops containing the water-soluble monomer are dispersed in the organic phase by stirring. The capsule membrane is then formed by adding the other organic solvent-soluble monomer to the continuous organic phase [141].

Polyelectrolyte complex: This method is formed by mixing polysaccharides of opposite charge, with a high potential for using in drug delivery systems and as well as in various biological and biotechnological applications. The characteristics of the polyelectrolytes, such as charge density, chain conformation, response to variation in pH, ionic strength and temperature, offer a wide set of variables that can be use to tailor the capsules to be used in different applications such as pharmaceutical formulations and cell encapsulation [66].

Ionotropic gelation: This is a very mild process formed with a variety of counterions or polymers, such as alginate, carrageenan, carboxymethylcellulose, pyrophosphate, octylsulphate, etc. [29, 178], where, the counterion polymers are prepared by reacting the polymer with divalent ions such as calcium [29].

1.1.2.4 Cell encapsulation methods

Cell encapsulation technology has the potential to treat a wide range of diseases by the controlled and continuous delivery of biological products to the host [176]. The aim of encapsulation is the immunoisolation of cells (avoiding immunologic response). The technique consists on surrounding cells with a thin microporous semipermeable membrane. The principle of encapsulating cell is that the permeability of the membrane is engineered to allow the efficient passage of oxygen, important nutrients and cellular products [179].

Many types of natural and synthetic polymers are being explored for cell encapsulation application, but the development of an ideal material to form a capsule is challenging. The majority of the literature describes the use of sodium alginate (polyanion) and poly-L-lysine (PLL-polycations) [136].

The polymers used for cell encapsulation must have specific characteristics such as, mechanical resistance, degradability, permeability, solubility and transparency [4, 129, 136], but the currently available polymers need to be improved by altering their surface and bulk properties in order to meet those requirements. The design of macromolecules must therefore be carefully tailored in order to provide the combination of chemical, interfacial, mechanical and biological functions necessary to manufacture new and improved biomaterials [4, 129].

Polyelectrolyte gels and polyelectrolyte complexes have played an important role in the microencapsulation of cells, with alginate and alginate/poly(L-lyine) complexes representing the most widely studied materials for these application. In the case of ionic cross-linking (figure 1-10), cells are first suspended in a polymer solution (e.g. sodium alginate), which is subsequently dropped into a receiving bath containing a multivalent cation (e.g. Ca^{2+}, Ba^{2+}) to create beads. This gentle gelation process is cell-compatible. Additionally, a polycation is used to form the outer layer to the beads (e.g. chitosan, poly(L-lysine), which often provides additional mechanical integrity [178] to the capsules.

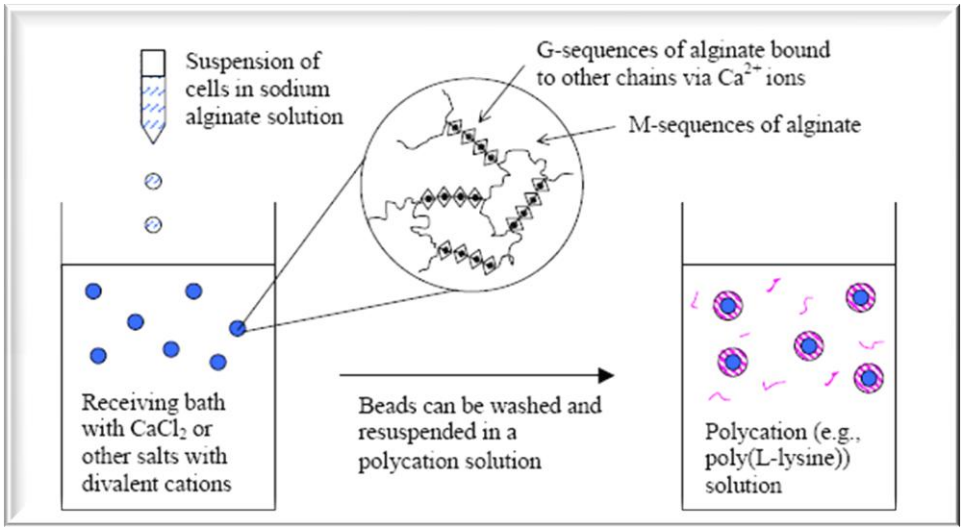

Figure 1-10. Schematic representation of a typical encapsulation process involving ionic cross-linking of alginate. Adapted from [178].

A wide variety of polyelectrolyte gels and complexes have been investigated for cell encapsulation. The Table 1-4 lists some physical characteristics of polyelectrolyte commonly used in cell encapsulation applications [178].

Table 1-4. Characteristics of some polyelectrolytes used for cell encapsulation. Adapted from [178].

Polyelectrolyte	Reported Characteristics	Ref.
Alginate	*Strong polyanion*. *In vivo* and *in vitro* applications with long-term of cell viability.	[124, 138, 180, 181]
Carrageenan	*Strong polyanion*. *κappa* and *ιota*-carrageenan form thermoreversible gels on cooling in the presence of appropriate counterions. Microbial cell viability.	[31]
Poly (styrene sulphonate)	*Strong polyanion*. A process for depositing alternating layers and microencapsulation. Cell viability and functionality was maintained.	[182, 183]
Carboxymethlcellulose	*Polyanio*n. A sign of insufficient biocompatibility was suppressed by altering the external surface material from Alg to CMC.	[184]
Cellulose sulfate	*Polyanion.* Preclinical studies of the biological properties, being developed for use as a topical contraceptive antimicrobial agent.	[26]
Heparin	*Polyanion*. Heparin is a highly sulfated glycosaminoglycan widely used as an injectable anticoagulant. It is also used to form an inner anticoagulant surface on various experimental and medical devices.	[185, 186]
Poly (methylene–co-guanidine)	*Polyanion*. Microcapsule has been developed based on the polyelectrolyte complexation which was successfully tested in rodent animal models.	[187]
Poly (diallyldimethyl ammonium chloride)	*Strong polycation*. Microcapsules developed by polyelectrolyte complexes, suitable for the encapsulation of these biological substances.	[188]
Chitosan	*Weak polycation*, pKa of primary amine 6.3-6.8 Polymer used for membrane's capsules.	[31, 189]
Poly (L-lysine)	*Weak polycation*, pKa of primary amine ~ 10.5 A novel technique for creating supported lipid membranes on polyelectrolyte multilayers is described. Encapsulated CHO cells 90% viability.	[190, 191]
Poly (vinylamine) hydrochloride	*Weak polycation.* Mechanically stable microcapsules have been produced for applications involving living cells and controlled delivery.	[192]
Poly (allylamine) hydrochloride	*Weak polycation*, pKa of amine group ~ 8.5 After encapsulation, cells preserve their metabolic activities and they are still able to divide.	[182]

1.2 BIOCOMPATIBILITY

One of the main aims of this thesis was to study the biocompatibility of chitosan based membranes and scaffolds as well as to assess the functionality i.e., the enhancement of cell adhesion on the surface of chitosan membranes due to several different plasma surface treatments.

Therefore, in this section, it will be described the importance of biocompatibility testing and the main types of cytotoxicity/biocompatibility assays that can be used to assess the biological behaviour of materials being developed for biomedical applications.

Biocompatibility of polymeric materials refers to the reaction of polymers with blood and tissue, depending on the site and purpose of use [126, 145]. Biocompatibility must be articulated within the context of an end-use application and has measurable dimensions only within this context [193].

The terms “biocompatibility assessment” and “safety assessment” have been generally considered to be synonomous. The safety assessment of biomaterials, prostheses, artificial organs and other medical devices generally is considered to be the determination of the biological interactions of the medical device in an *in vivo* environment [194]. The goal of safety testing is to evaluate if a medical device or biomaterial support presents potential harm to the patient or user under conditions simulating use. A broad definition of biocompatibility applied to tissue engineering implants includes the determination of the biological interactions of the device, i.e. biomaterial component, tissue component, and combination of biomaterial and tissue components, in an *in vivo* environment [194, 195].

One of the major problems in the use of polymers as biomaterials is to make sure that they are biocompatible by themselves, and that the use of particular additives and/or processing technologies required to obtain different properties and or configurations will not interfere with the biocompatible behaviour [196, 197].

In terms of biocompatibility, the requirements that degradable materials must fulfil are much more demanding than those for non-degradable materials. In addition to the potential problem of toxic contaminants leaching out from the implant, such as residual monomers, stabilisers, emulsifiers and many other types of additive, it is also

necessary to considerer the potential toxicity of the degradation products and subsequent metabolites [198]. Natural origin polymers, besides being biodegradable, present some characteristics that may affect their biocompatibility behaviour.

In fact, these polymers usually contain domains that can send important signals to guide cells at various stages of their development [175, 199, 200]. However, this bioactivity can cause problems with antigenicity [175, 200], because when the material is implanted in a host, this might stimulate an immune response, potentially leading to immune rejection. Additionally, the degradation of natural polymers often relies on enzymatic processes that produce variation in the degradation rate. Nevertheless, natural origin polymers were the first to be used as scaffold materials for tissue regeneration [175, 200].

1.2.1 *IN VITRO* CYTOTOXICITY/BIOCOMPATIBILITY ASSAYS

Cytotoxicity testing represents the initial phase in testing the biocompatibility of potential biomaterials and medical devices. Its purpose is to act as a reliable, convenient, and reproducible screening method to detect, at an early stage in the testing process, cell death or other serious negative effects on cellular functions [201-205].

The term “cytotoxicity” means to cause toxic effects (death, alterations in cellular membrane permeability, enzymatic inhibition, etc.) at the cellular level. It is clearly different from physical factors that affect cellular adhesion (surface charge of a material, hydrophobicity, hydrophilicy, etc.) [206]. A toxic material releases a chemical in sufficient quantities to affect cells directly or indirectly through inhibition of metabolic pathways [206].

Cytotoxicity *in vitro* assays are referenced in national and international standards [194, 206, 207]. Such methods can be applied to both direct and indirect testing assays. These assays differ mainly in the manner in which the test material is exposed to the cells. The choice of the method varies with the characteristics of the test material, the rationale for doing the test and the application of the data for evaluating biocompatibility [206, 208, 209]. Regarding indirect contact two methods can be considered [208, 209]. The first consists of the separation of the material from cells by

a diffusion barrier, such as an agar layer, place between the material and a cell monolayer. The second consists of the addition of an extract of the material to a cell monolayer. Indirect testing techniques are important for the detection of leachable substrates, which could exert toxic effects on cells [208]. Direct contact techniques involve assays in which the material to be tested is brought into direct contact with the cells, usually by seeding a cell suspension onto the material [208, 209] (Figure 1-11).

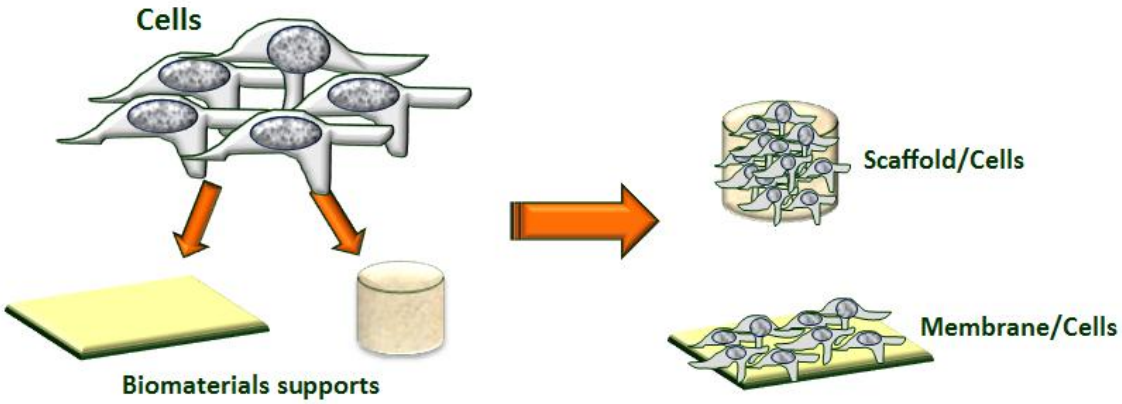

Figure 1-11. Schematic representation of the set up for a Direct Contact Assay, where the cell suspension is seeded onto a membrane or scaffold materials.

In the direct contact assay, the interaction cell-material is very important because this interaction allows the adherence and attachment of different cell types on the surface of the material (2D or 3D).

Adhesion of cells (mammalian and microbial) to surfaces is a complex, multi-steps process. Initial bio-adhesive steps involve transport of the cells into the inter-phase that separates a surface from the suspending medium through mass-transfer processes, such as flow or gravitation [193].

1.2.1.1 Assessment of cell attachment and adhesion

Cell adhesion is probably the single most important aspect of cell interaction with a biomaterial because it is a prerequisite for the further cellular functions such as spreading, proliferation, migration and biosynthetic activity [196, 208]. Indeed, surface

characteristics of materials (topography, chemistry, surface energy, wettability) play an essential role in cell adhesion onto biomaterials [4, 193, 196, 210].

Attachment is different than adhesion. Adhesion refers to the force necessary to separate adherents and is controlled by short-range forces resulting from the formation of covalent, ionic, hydrogen and charge-transfer bonds. Thus, adhesion occurs only if cellular surfaces (or some extension of the cell surface) touch the substratum so that atomic-level contacts can be made. In contrast, attachment can be of the physical kind in which cells are held in an attractive potential well near the substratum, formed through a conspiracy of electrostatic repulsion and dispersion force attraction. Attachment occurs first, then possible adhesion. Once attached to a substratum cells can spread and proliferate on this surface [193].

Initial cells/materials interactions can be characterized in four stages: 1) protein adsorption to the surfaces, 2) contact of rounded cells, 3) attachment of cell to the substrate, and 4) spreading of cells. Initial cell attachment depends mainly on the substrate characteristics and cell type [211, 212].

Diverse biological responses to materials, such as protein absorption and cell adhesion respond to local solvent properties of water against hydrophobic and hydrophilic surfaces [193]. In the case of protein as solute can be adsorbed to hydrophobic surfaces but not to hydrophilic surfaces, because surface dehydration is energetically unfavourable. Previous studies suggest that proteins can be associated or entrapped within bound-water layers against hydrophilic surfaces in a fashion that does not formally involve surface dehydration. Additionally, hydrophilic surfaces support attachment of mammalian cells and efficiently active the blood plasma coagulation cascade through mechanisms that apparently do not involve surface-adsorbed protein [193].

1.2.1.1.1 Surface treatment. The surface of biomaterials plays a vital role in biomedical applications. In the case of polymers, these often do not have the adequate surface properties for biomedical applications [213, 214], although, they have excellent bulk physical and chemical properties [214]. Therefore, polymers have been submitted to several treatments in order to enhance their properties, particularly to increase cell adhesion and proliferation onto their surfaces. In recent years, many advances have

been made in developing surface treatments to alter the chemical and physical properties of polymer surfaces without affecting bulk properties. Common surface modification techniques include treatments by flame, corona, plasmas, photons, electron beams, ion beams, X-rays, and γ-rays [214]. Plasma processes as surface treatment techniques are becoming more common in biomaterials engineering [210]. Plasma treatment is probably the most versatile surface treatment technique. Different types of gases such as argon, oxygen, nitrogen, fluorine, carbon dioxide, and water can produce the unique surface properties required by various applications [210, 214, 215]. For instance, nitrogen-containing plasma is widely used to improve wettability, bondability and biocompatibility of polymer surfaces. Ammonia and nitrogen plasma have been used to provide surface amino binding sites for immobilization of heparin on a variety of polymer surfaces [214]. In the biomedical area, plasma treatments have been used to modify biomaterials for various applications including devices or implants for diagnosis or therapy such as contact lenses, artificial heart valves, vascular grafts, catheters, dialysis membranes, prosthetic devices, and materials for bone joint repair and replacement [216].

Plasma deposition (including both grafting and polymerization) has been utilized to create a new surface chemistry for enhancing the culturing of cells on surfaces, for modifying protein and cell interactions, or to reduce surface friction and thereby enhance the biocompatibility of the material; to create a barrier film in order to reduce undesirable diffusion of small molecules into or out of the substrate (e.g. absorption from the environment and release of leachables from the substrate), or to control the rate of diffusion of drugs from the substrate; and to form reactive groups on the surface for subsequent immobilization of biomolecules or for grafting and polymerization with other chemical compounds [214, 217, 218]. The capability of plasmas to alter surface physical and chemical properties without affecting the bulk properties (especially mechanical properties) of the base material is advantageous in the design, development, and manufacturing of biocompatible polymers. By either surface modification or thin-film deposition, specific surface chemistries can be created for optimization of protein-surface interactions and therefore biocompatibility (especially blood compatibility) of biomaterials (Figure 1-12) Plasma etching

treatments are applied to biomedical material surfaces for sterilization, wettability improvement, providing reactive sites, and crosslinking surface molecules [214].

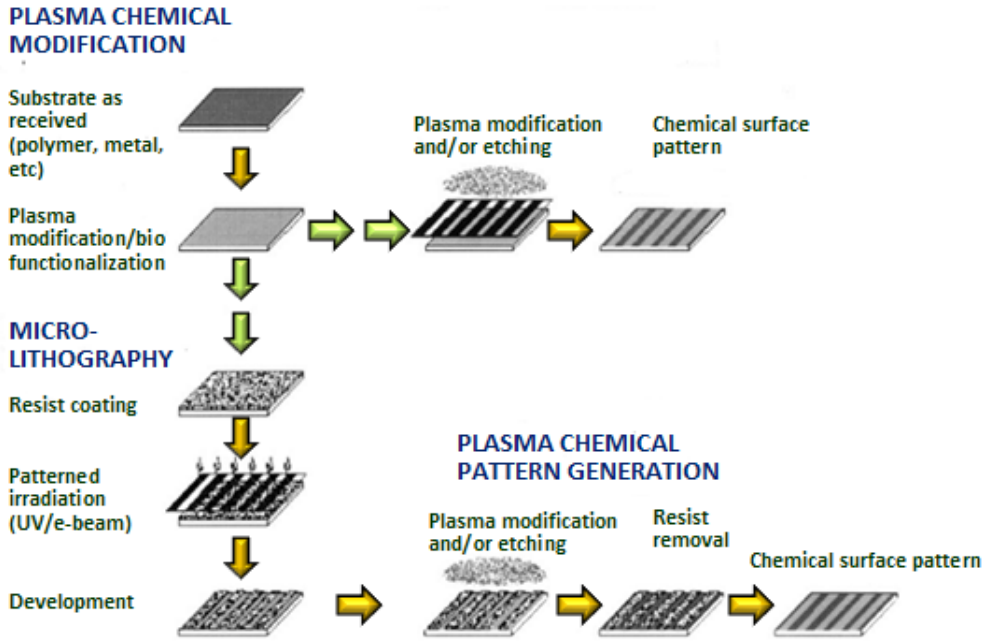

Figure 1-12. Possible processing procedures for plasma-induced chemical micropatterning. Adapted from [215].

Particularly in tissue engineering, as previously mentioned, it is often necessary to modify the surface of the scaffolds developed for cell attachment and growth. For this purpose, there are three basic approaches that can be considered (Figure 1-13).

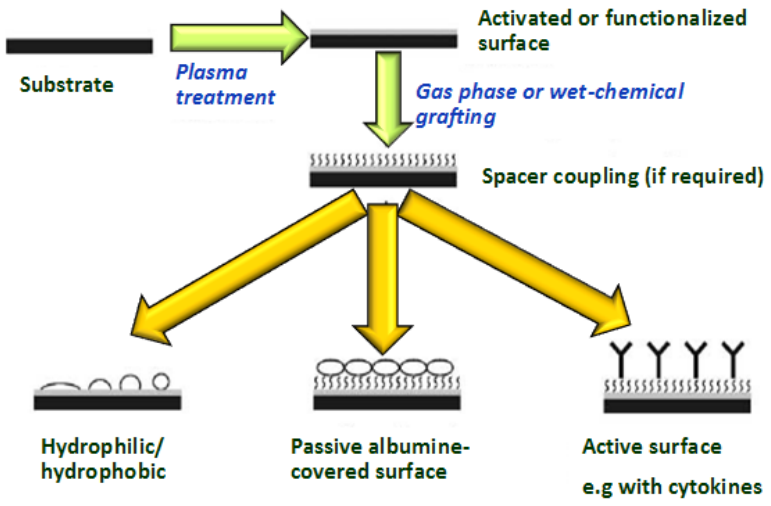

Figure 1-13. Approaches for enhancement of cell growth on surfaces. Adapted from [145].

First, the surface tension may be adapted by adding chemical groups to the surface which lead to a defined hydrophilicity. Second, amine or carboxylic functions used to bond proteins or protein segments can be provided, so that the cells will not perceive the surface to be ‘‘foreign’’. Third, so-called growth factors (cytokines) can be bonded to the surface, so as to accelerate cell growth. A cell growth improvement on a plasma-treated technical material can be developed for various cell types, e.g. for skin cells (keratinocytes) or cornea cells of the eye. Possible applications are the production of artificial skin (e.g. for burn victims and in cosmetic tolerance analysis), artificial cornea cells can be used as a substitute for animal experiments [145].

In this work, and in order to improve cell adhesion and proliferation, surface modification of chitosan based membranes were carried out by plasma using gases such as nitrogen and argon.

1.2.1.2 Relevance of *in vitro* testing and cell types used in biocompatibilty studies

The major advantage of using *in vitro* methods for cytotoxicity/biocompatibility testing is their cost effectiveness and speed, which make them particularly suitable for screening large numbers of potential biomaterials and their modifications [204, 219]. Additionally, animal experiences are reduced. Coupled with this is the high sensitivity of the methods, which enables potentially cytotoxic materials to be identified at an early stage in the testing procedure [202, 220].

The fundamental problem of *in vitro* methods is the need to extrapolate to the *in vivo* situation [219, 221]. However, this connection is far to be established and thus it should be stressed that *in vitro* testing represents only one phase in assessing biocompatibility [221]. Specimens classified as *in vitro* biocompatibility must enter a further phase of testing, which requires *in vivo* observation [222].

Cell culture methods have been used to evaluate the biological compatibility of materials for more than two decades. Most of the cells used for culture assays are cell lines previously established that can be purchased from biological suppliers or cell banks. These cells offer higher reproducibility, efficiency and in some cases availability in comparison with primary cells or other cell types [206].

Cell lines have been developed for growth *in vitro*. The use of these cells in biocompatibility tests is preferred to primary cells (that are freshly harvested from live

organisms) because the cell lines improve the reproducibility of the assays and reduce the variability among laboratories. Additionally, cell lines maintain their genetic and morphological characteristics throughout a long life period [206]. However, the use of a cell line can be considered partially transformed cells with a predisposition to become fully neoplastic cells capable of forming tumours in the recipient [211].

The L-929 mouse fibroblasts cell (Figure 1-14, C-D) has been one of the most extensively used for testing biomaterials. Initially, L-929 cells were selected because they were easy to maintain in culture and produced results that had a high correlation with specific animal bioassays. Furthermore, fibroblast was chosen because it is one of the early cells to populate a healing wound and is often the major cell in the tissue that attaches to implanted medical devices [206, 223]. Cells lines from other tissues or species may also be used such as SaOS-2 Human osteosarcoma cell line [224] (Figure 1-14, A-B), ATCC murine hybridoma cell line HB121 [140], 1B5 hybridoma cell line [8], MDCK cells and canine renal epithelial cell line [139], etc. Selection of a cell line is based mainly on the type of assay, type experience and specific end application, among others various factors. Positive and negative controls are often included in the assays to ensure the operation and suitability of the test system [206].

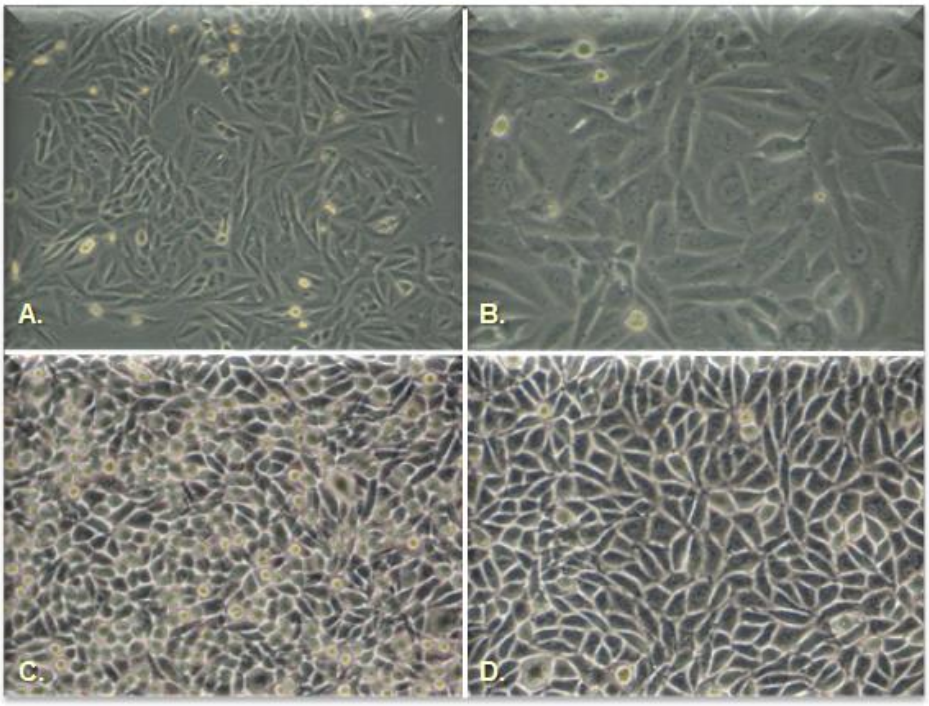

Figure 1-14. Morphology of some cell lines used in biocompatibility tests. A and B images showed SaOS-2 cell line; C and D images showed L929 cell line. All photos were taken using inverted microscope using the objectives 10X (left images) and 20X (right images).

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CHAPTER 2

2 MATERIALS AND METHODS

This thesis comprise two major studies, the first corresponds to the study of the biocompatibility of chitosan membrane and scaffolds and the second to the development of a new cell encapsulation system based on alginate and carrageenan polymers. This chapter describes, in more detail, the materials and techniques used.

2.1 MATERIALS DEVELOPMENT AND PREPARATION

2.1.1 CHITOSAN MEMBRANES

The chitosan membranes studied were obtained by solvent casting technique as described previously [1]. Briefly, Chitosan (powder) were dissolved in aqueous acetic acid 2% (v/v) solution at a concentration of 1% wt. Then the chitosan solution was casted into a Petri dish and dried at room temperature for about 6 days. In order to neutralize the acetic acid, the dried membranes were immersed in 0.1 M sodium hydroxide for about 10 min, and then washed with distilled water to remove all traces of alkali, followed by drying at room temperature. The developed membranes were then submitted to plasma surface treatments, performed under different conditions, in order to select the parameters that lead to best results in terms of improving cell adhesion and proliferation. The plasma treatment was carried out on a plasma reaction equipment (PlasmaPrep5, Germany), using a power of 20 W. The duration of the glow discharge treatment was varied from 10 min to 40 min, using nitrogen and argon gas. The plasma conditions tested and the correspondent samples designation used are presented in Table 2-1. Untreated chitosan membranes were used as control and designated by Cht.

All the membranes submitted to plasma surface modification treatments were cut into squares of 1x1 cm^2 and sterilized by ethylene oxide prior to all the biological studies performed.

Figure 2-1. Chitosan membrane

Table 2-1. Plasma conditions used to treat the surface of chitosan membranes

Sample	Conditions		
	Power [watts]	Time [min]	Gas
ChtP1	20	10	Nitrogen
ChtP2	20	20	Nitrogen
ChtP3	20	30	Nitrogen
ChtP4	20	40	Nitrogen
ChtP5	20	10	Argon
ChtP6	20	20	Argon
ChtP7	20	30	Argon
ChtP8	20	40	Argon

2.1.2 CHITOSAN SCAFFOLDS

The chitosan scaffolds studied in this thesis were developed by means of combining a sol-gel process and the freeze-drying technique described previously [2, 3]. Briefly, the chitosan powder (Sigma, Portugal) was dissolved in acetic acid 2 % (v/v) at a concentration of 4 % wt. The obtained chitosan solution was poured into a Petri dish and allowed to dry at room temperature for several days. By its turn, the porous hybrids were obtained by transferring the mixture into silicon cilindrical moulds, freezing at -80 ^{o}C overnight, followed by free-drying for the period of 4 days to completely remove the solvent [2]. The scaffolds were neutralized with sodium hydroxide (NaOH, Panreac) solution for 10 min and washing abundantly with distilled water until reaching the pH 7.0. Afterwards the scaffolds are placed again in the freezing at -80^{o}C overnight, followed by free-drying for the period of 2 days [2]. Table 2-2, showed the conditions used in the preparation of the several chitosan porous scaffolds formulations. After samples processing, the scaffolds were cut into cylinders of 0.6 cm (b) x 0.3 cm (h) x 0.25 cm (r) and sterilized by ethylene oxide prior to testing (Figure 2-2).

Table 2-2. Conditions used in the preparation of the several chitosan porous hybrid scaffolds

Sample	polymer (g)	TEOS:HCl (wt%)	$CaCl_2$ (g)	Reaction time (hours)
CT5	79.2	0.1:0.1	-	72
CT6	79.2	0.1:0.1	0.0788	72
CT7	79.2	0.1:0.01	-	72
CT8	40	0.1:0.1	-	24

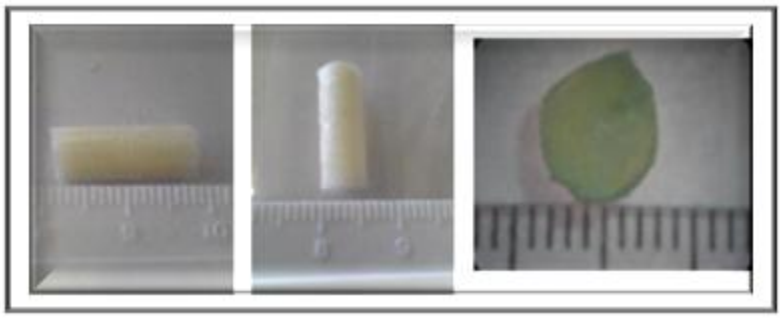

Figure 2-2. Chitosan scaffolds

2.1.3 DEVELOPMENT OF CARRAGEENAN AND ALGINATE BASED MICROCAPSULES

2.1.3.1 Development of microcapsules based on *iota* and *kappa*-carrageenan

The microcapsules based on *iota* (Sigma, Portugal) and *kappa*-carrageenans (Sigma, Portugal) were developed from a pair of oppositely charged polysaccharides. For this purpose, 1 ml of an 1.5 % aqueous solution of *iota* (or *kappa*)-carrageenan was added drop by drop through a 0.3 mm needle (BD-Microfine U-100 insulin, Portugal), using a syringe pump (AL-1000, Aladdin Programmable Syringe Pump) into 20 ml of an 0.15 % chitosan low molecular weight solution (0.0085 M HCl, Aldrich, Portugal). Both polysaccharides were dissolved in distilled water [4]. Capsules were also obtained using chitosan solutions at two different pHs (5.0 and 6.0) previously adjusted with β-Glycerophosphate (β-GP, Sigma-Portugal) [5] as described below.

The resulting capsules were allowed to harden for 45 min at 37 ^{o}C (in a water bath), washed with 0.9 % NaCl and immersed in a 0.05% *iota*-carrageenan solution for 5 minutes in order to link the remaining residues of chitosan. Finally, the capsules were rinsed twice with PBS and stored at 4 ^{o}C in the same buffer (Figure 2-3).

Adjustment of the pH of the chitosan solution using β-glycerol phosphate

Chitosan solutions, with different pHs were used in order to form different types of capsules by polyelectrolyte complex and ionotropic gelation method [6]. In both methodologies, chitosan solution was used as polycation to obtain the membrane of the capsule at two different pHs: pH 5.0 and pH 6.0. Previous studies have demonstrated that chitosan can be neutralised up to physiological pH (7.2) using β-

glycerol phosphate (β-GP), without promoting the precipitation of the polysaccharide [5, 7].

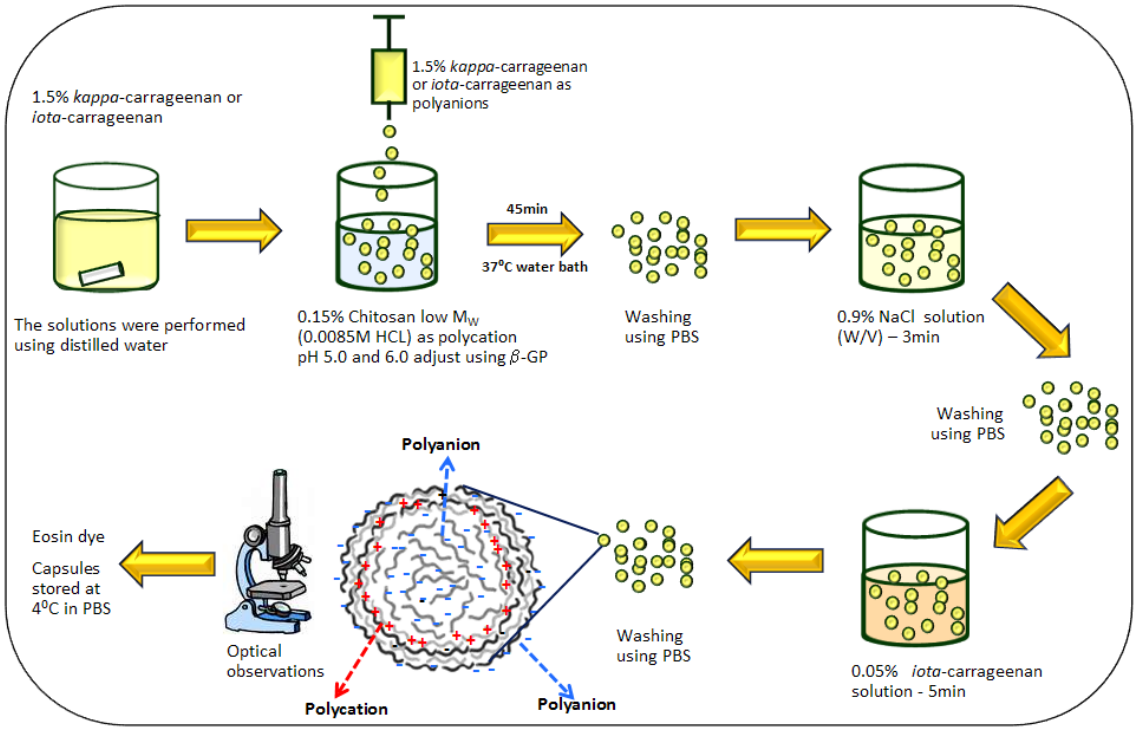

Figure 2-3. Graphical representation of the procedure used for the capsules development using oppositely charged polysaccharides (carrageenan as polyanion and chitosan as polycation).

A curve of the variation of the pH in chitosan solution as function of β-GP concentration was drawn to determine the correct amount of β-GP to be added to reach the desired pH. For this purpose, a solution containing 0.0266 g/ml of β-GP was slowly added to a chitosan solution (0.30 % in 0.017 M HCl) until physiological pH. At this point (pH = 7), it was observed precipitation. The obtained curve is presented in Figure 2-4.

2.1.3.2 Development of sodium alginate/*iota*- carrageenan microcapsules

Microcapsules based on *iota*-carrageenan and alginate were developed by an ionotropic gelation method that was originally developed for the preparation of alginate capsules [6, 8, 9]. The procedure starts with the preparation of an aqueous sodium alginate/*iota*-carrageenan mixture composed of 1.5% aqueous solution of each polymer, at two different ratios, namely 50/50 % and 30/70 % (v/v), in order to determine the best formulation. Then, 1 ml of this mixture was extruded through the

0.3 mm needle, using a syringe pump, into 20 ml of a solution composed of 0.05 M $CaCl_2$ and 0.2 M NaCl. All solutions were prepared in distilled water.

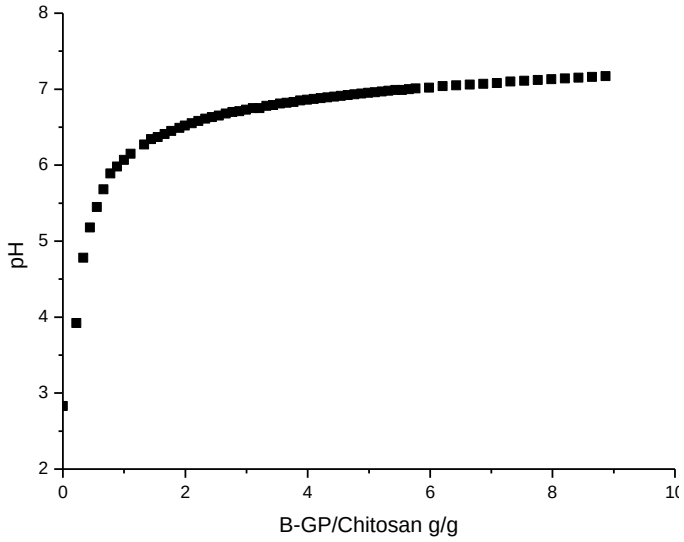

Figure 2-4. Variation of the pH relative to amount of β-GP with respect to chitosan (weight β-GP/weight chitosan g/g), at room temperature

The resulting microcapsules were allowed to harden for 30 min at 37 ^{o}C. Afterwards, the capsules were immersed in 20 ml of a 0.15 % (w/w) chitosan low Mw solution (in 0.0085 M HCl) at two pHs (pH 5.0 and 6.0) previously adjusted with β-glycerophosphate (as was describe in section 2.1.3.1) for 20 min at 37 ^{o}C. After this, the capsules were rinsed with PBS for 1 min and immersed in a 0.05% *iota*-carrageenan for 5 minutes. Microcapsules with liquefied/depolymerised core also were prepared using a sodium citrate (a calcium sequestering agent) treatment. This treatment consisted in the immersion of the obtained *iota*-carrageenan + alginate microcapsules in a 0.055 M solution of sodium citrate for 1 min [10, 11]. Finally, the obtained capsules (solid and liquefied) were rinsed twice with PBS buffer and stored at 4^{o}C in the same buffer for posterior characterization (Figure 2-5). Another batch of capsules were immersed in DMEM to assess the stability of the microcapsules in culture medium and also to observe the permeability of the culturing medium into the capsules.

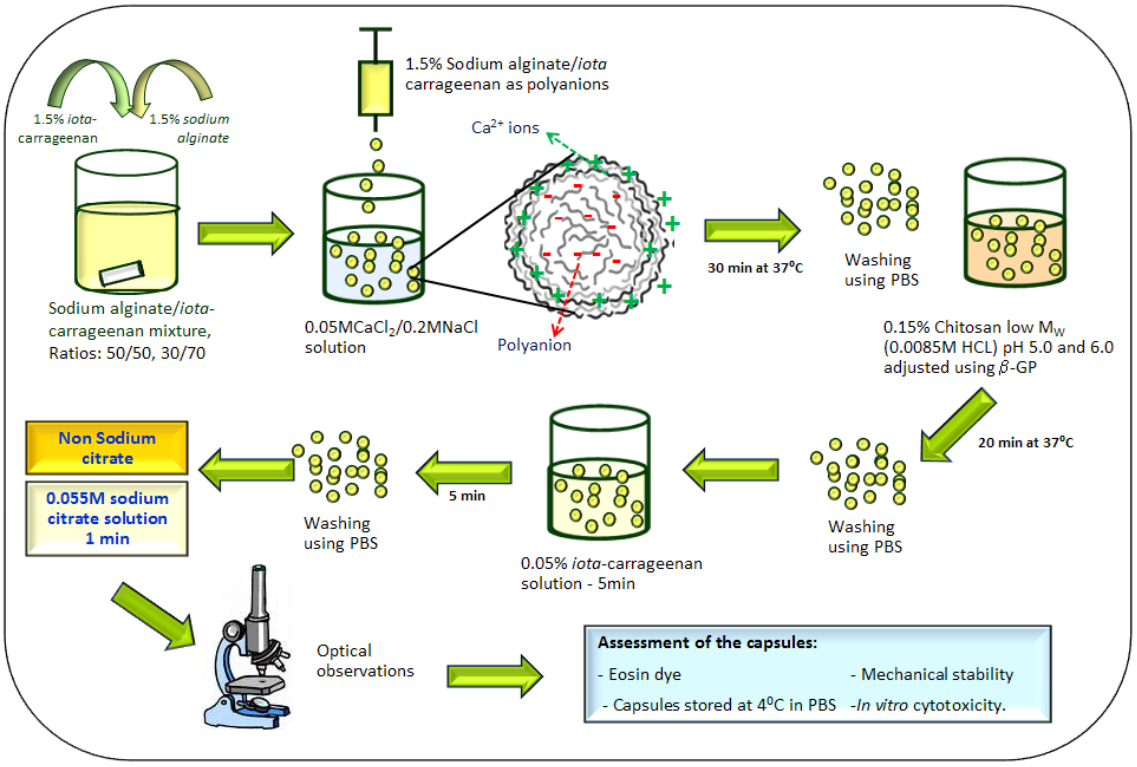

Figure 2-5. Graphical representation of capsules produced by ionotropic gelation method

2.1.3.3 Cell encapsulation studies

Immobilisation of the cells was performed using a L929 fibroblasts cell line in sodium alginate/*iota*-carrageenan capsules (solid and liquefied beads). Microcapsules and cell encapsulation procedures were carried out at room temperature under sterile conditions, in a laminar flow cabinet. All solutions were prepared in distilled water and filtered using a membrane filter of 0.2 µm (Schleicher & Schuell Microscience, Germany). Before using in cell encapsulation experiments, the sodium alginate, *iota*-carrageenan and chitosan solutions were autoclaved. The fibroblasts cells were initially harvested from monolayer using trypsin-EDTA (Invitrogen, Portugal), and a cell suspension containing a know amount of cells was prepared. The cells ($5x10^3$ cells per ml) of the polymeric solution were then mixed with the sodium alginate + *iota*-carrageenan solution (30/70%). The cells-polymeric solution suspension was extruded into a calcium chloride-sodium chloride ($CaCl_2$-NaCl), solution, as described above (section 2.1.3.2) using a syringe pump. The resulting microcapsules/cells were maintained in $CaCl_2$-NaCl for 30 min at 37 °C in a 5 % CO_2 incubator. Afterwards, the capsules containing the cells were immersed in 20 ml of 0.15 % (w/w) chitosan low Mw

solution (in 0.0085 M HCl) at pH 5.0 for 10 min and at 37 ºC. After this, the capsules were rinsed with PBS for 1 min and immersed in 0.05% *iota*-carrageenan for 5 minutes. To obtain the liquefied capsules, the microcapsules with cells were treated with 0.055 M of sodium citrate for 1 minute. Finally, microcapsules with cells were washed twice with PBS, transferred to several wells of a 6-well culture plate, and immersed in culture medium (Figure 2-6). The encapsulated cells were cultured in a 5 % CO_2 incubator for different periods of time, namely 1, 2, 3 and 7 days. The culture medium was carefully changed every 2 days.

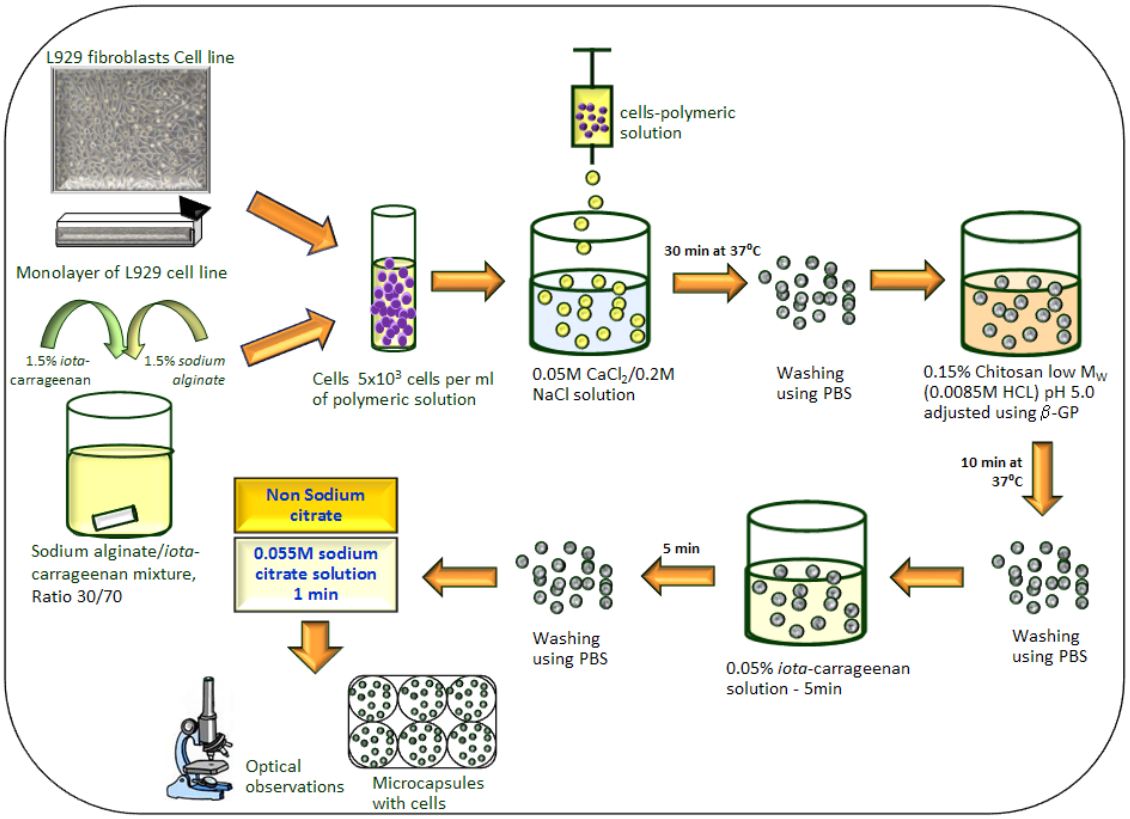

Figure 2-6. Graphical representation of the cell encapsulation procedure carried out using the ionotropic gelation method.

2.2 CELL CULTURE STUDIES

2.2.1 CELLS USED

For most of the cytotoxicity/biocompatibility tests performed, it was used the mouse lung fibroblast (L929) cell line, obtained from the European Collection of Cell

Cultures (ECC, UK). This cell line was grown as a monolayer cultured in 75 cm^2 culture flasks, using Dulbecco's Modified Eagle's Medium-low glucose (DMEM; Sigma-Aldrich, Portugal), supplemented with 10 % foetal bovine serum (FBS; Biochrom AG; Germany), 1 % antibiotic/antimycotic (Invitrogen, Portugal) and sodium bicarbonate (Sigma, Portugal) at 37 ^oC in a 5 % CO_2 incubator [12]. The culture medium was changed every 2 days and cells were grown until confluence, before starting each *in vitro* test. This cell line was selected for these studies because it is commonly used for cytotoxicity assessments related with biomaterial applications [12-18] as these cells are able to maintain their phenotypic characteristics for long periods in culture [19].

Further studies, namely for the assessment of cell adhesion and proliferation on chitosan scaffolds, it was used a cell line of osteoblastic like cells-SaOS (human osteosarcoma), obtained from European Collection of Cell Cultures (ECC, UK). As for the L929 cell line, SaOS cells were grown as monolayer in cell culture flasks using DMEM, supplemented with 10 % FBS and with 1 % of antibiotic/antimycotic mixture, and trypsinized prior to use. The chitosan scaffolds are aimed to be applied in bone tissue engineering and, for this reason, we have selected these cells. Previous studies have shown that these cells express the same range of extracellular matrix proteins as native osteoblasts [20, 21].

2.2.2 CYTOTOXICITY TESTING

Cytotoxicity testing represents the initial phase in testing biocompatibility of potential biomaterials and medical devices [22-27]. For this work, the cytotoxicity of leachables of some biomaterials was evaluated using two cell culture methods, namely MEM extraction test (Minimum Essential Medium, 72 h-short term) and MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, both carried out after a 24 hours extraction period, according to protocols described in ISO/EN 10993 [28]. Latex rubber (Dermagip, WRP) was used as a positive control and standard culture plates (Costar 96 well plates) were used as negative control. The biocompatibility of the chitosan membranes was assessed using the MEM and MTT test and the cytotoxicity of sodium alginate/*iota*-carrageenan capsules was evaluated by MTT test.

2.2.2.1 Extraction Procedures

The extracts of materials (and positive control material), were obtained by immersion of materials in culture medium for 24 hours at 37 ºC, 60 rpm. In the case of chitosan membranes, 60 samples of 1 cm^2 (of each formulation) were immersed in 50 ml sterile tubes containing 10 ml of culture medium (DMEM-the same used for cell culture) and placed in a water bath at 37 ºC and 60 rpm for 24 hours (Figure 2-7). In the case of the microcapsules, the extracts materials were obtained by immersion of 100 microcapsules (of each type) in a sterile tube containing 5 ml of DMEM and placed in a water bath at 37 ºC and 60 rpm for 24 h. In both cases, latex rubber was used as positive control, where the latex extracts were obtained by immersing 60 latex squares of 1 cm^2 in sterile tubes containing 10 ml of DMEM, following the same procedure as described for obtaining the membranes and capsules extracts. After the 24 h extraction time, the tubes were removed from the water bath and their content (extract material) was filtered using a membrane filter of 0.45 µm (Schleicher & Schuell Microscience, Germany) and transferred to new sterile tube.

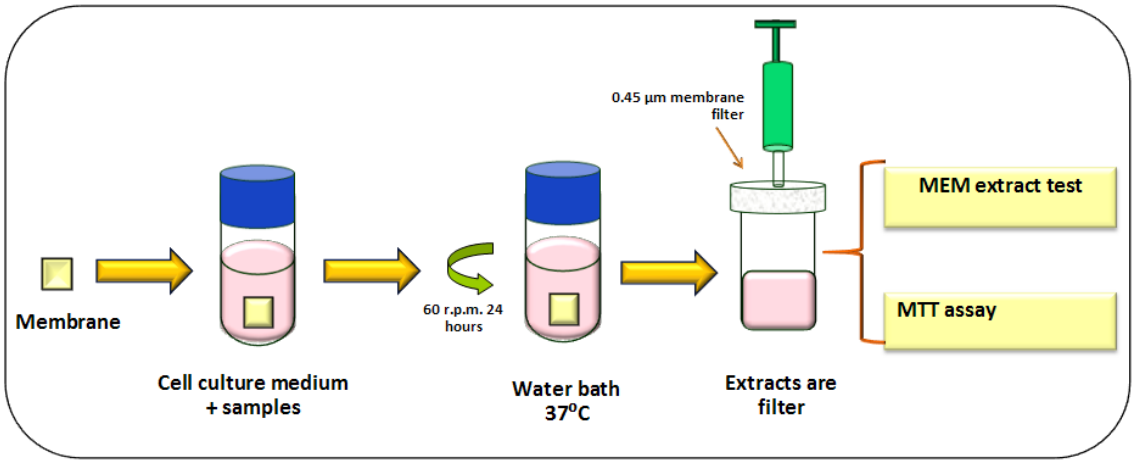

Figure 2-7. Graphical representation of the procedure used to prepare extracts of the chitosan membranes

2.2.2.2 MEM extraction test (72h)

The MEM (Minimum Essential Medium) extract test is a cytotoxicity test that has as main objective evaluating qualitatively the rates of cell damage (change of cellular morphology, inhibition of cell growth, cellular lysis and confluence of monolayer).

For this test L929 cells were seeded in 6-well plate (n = 3) at a density of approximately 4.5 x 10^4 cell/ml when the cells reached about 80 % confluency (after 24 h of culture). The culture medium in each well was replaced by 2 ml of extraction fluid and the response of the cells was evaluated for 72 h. Morphological characteristics were evaluated after 24, 48 and 72 hours using an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306), and compared to the negative and positive control. The observations based on the confluency of the monolayer, degree of floating cells and changes in cellular morphology were scored, according to the parameters defined in Table 2-3 and as it has been reported in previous studies carried out in our group [12-14, 29].

After 72 h testing, the cells were detached from the culture wells by means of trypsinization. The percentage of growth inhibition was determined by cell counting, using a Neubauer camera and scored after correction for the value of the negative control. Addition of these two scores results in the final cytotoxicity response index ranging form 0 to 8 (see scores on Table 2-4) [12-14, 29].

Table 2-3. Scoring for morphological changes, confluence and cell death, used in the cytotoxicity tests

Score	Confluency [%]	Floating cells [%]	Change of cellular morphology	Inhibition of cell growth [%]
0-1	100	0	No changes during test period	0-10
1-3	90-100	0-5	Slight changes, few cells affected	10-30
3-5	60-90	5-10	Mild changes some cells round/spindle shaped	30-50
5-7	30-60	10-20	Moderate changes, many cells round/spindle	50-70
7-8	0-30	>20	Severe changes, about all cells show morphological changes	70-100

Table 2-4. Cytotoxicity indexes used to classify the reactivity of tested samples - cytotoxic response

Cytotoxic response	Reactivity	Pass/fail
0-1	None	Pass
1-3	Slight toxic	Pass
3-5	Mild toxic	Retest
5-7	Moderate toxic	Fail
7-8	Severe toxic	Fail

2.2.2.3 MTT test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-Sigma), is a yellow vital dye that is actively converted into blue formazan crystals by mitochondrial oxidation-reduction reaction. The formation of the blue formazan crystals formed within the cells is directly proportional to the viability of the cells [30]. The crystals formed intracellularly were dissolved by isopropanol [31].

In the MTT test, fibroblasts cells were seeded in 96-well plates (n = 6), at density of approximately 6.6 x 10^4 cell/ml after reaching about 80% of confluence (after 24 h of culture) and the culture medium in each well was replaced by 150 µl of extraction fluid (membranes extracts or capsules extracts) and the response of the cells was evaluated for 72 h. After this time, MTT solution was prepared (1 mg MTT/ ml in DMEM without red phenol and FBS) and 50 µl of this solution was added to the culture wells. The cultures were then incubated for 4h at 37 ^oC in the dark. Following this time, the MTT solution was removed and replaced by isopropanol (100 µl) for 15 minutes, in order to lyse the cells (the purple solution appeared). Finally, the optical density (OD) was measured in a microplate reader (BIO-TEK – Synergy HT) at 570 nm with a background correction of the OD at 650 nm [14]. The evaluation expresses the variation of the absorbance in function of the concentration of extracted material. The results were expressed as percentage of cell viability.

2.2.2.4 MTS test

Cellular viability and proliferation in chitosan based membranes and sodium alginate/*iota*-carrageenan capsules were assessed by the MTS using CellTiter 96® one cell proliferation assay (Promega, Madison, USA). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test has been described previously [15, 16, 32]: the substrate-MTS is bio-reduced into a brown formazan product by dehydrogenase enzymes, which are active in living cells. The intensity of the colour is directly related to the number of viable/living cells [15, 16]. In this study the MTS solution was prepared with DMEM without phenol red and FBS in a 5:1 ratio, where 300 µl of MTS solution was added to membranes/cells and incubated for 3 hours at 37 ^oC in an atmosphere containing 5% of CO_2 in the dark. To assess the viability of cells encapsulated into alginate/carrageenan capsules, 200 µl of MTS

solution was added to the capsules with cells (3 per 96 well-culture plate) and incubated for 3 hours at 37 ^{o}C, 5% CO_2 in the dark. In both cases, after incubation period, 100 µl of solution obtained from each membrane and capsules, were transferred to 96-well plates (in triplicate). The absorbance was read in a microplate reader (BIO-TEK – Synergy HT) at 490 nm. The results were expressed as absorbance obtained for the samples, in each culture period.

2.2.2.5 Direct Contact assay

The Direct Contact assay was used to evaluate the cell adhesion and cell proliferation on the surface of chitosan based membrane treated by plasma. For this purpose, L929 fibroblast cells were initially harvested from monolayer cultures using trypsin-EDTA. A cell suspension was prepared in order to obtain a cell density 8 x 10^4 cells/cm^2 [33]. The membranes were placed in 24 well plates and 100 µl of this cell suspension was seeded onto the samples surface (1x1 cm^2) (Figure 2-8). Empty wells and coverslips (Sarstedt, USA), were also seeded with the same amount of the cell suspension to be used as controls. All the seeded samples and controls were incubated for 2-3 hours at 37 ^{o}C in an atmosphere containing 5% of CO_2. After this time, 1.5 ml of DMEM culture medium was added into each culture well and the samples cultured for different periods of time (3, 7 and 14 days). The culture medium was changed every two days.

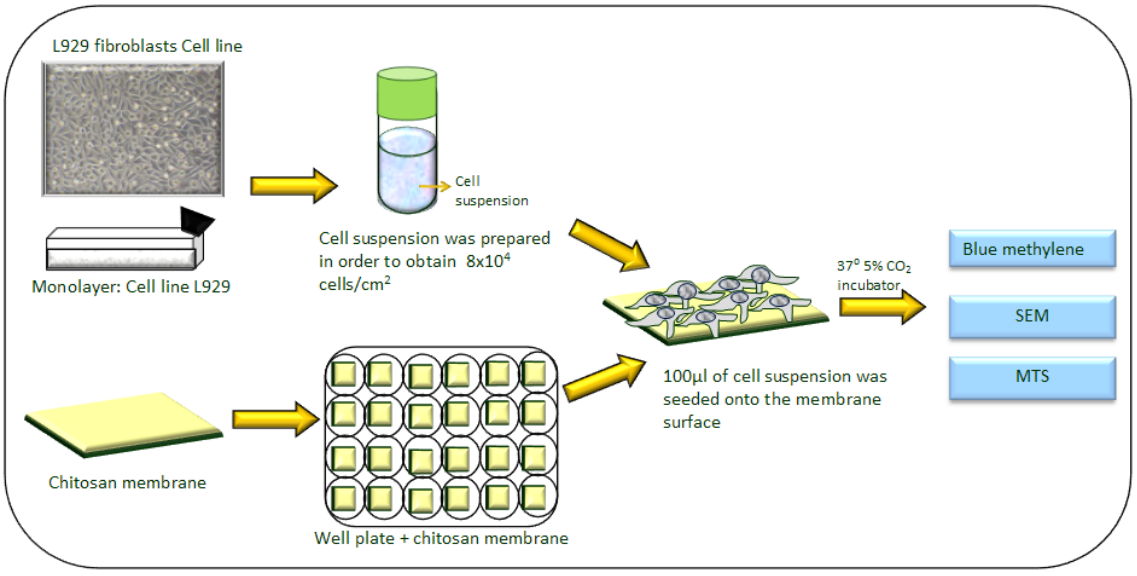

Figure 2-8. Graphical representation of the procedure used for cell seeding onto chitosan membranes

2.2.2.6 SEM analysis

Cell adhesion was assessed by SEM (scanning electron microscope) for all the tested chitosan based materials. This technique is particularly useful to observe cell adherence and morphology in the different substrates. In the case of chitosan based membranes, after each culturing period, the chitosan membranes were removed from culture and washed with PBS, before being dehydrated using an increased concentration series of ethanol solutions (25%, 30%, 50%, 70%, 80%, 90% and 100% - each 30 min). The membranes were then dried at room temperature (in the laminar flow chamber) and sputter coated with gold before observation in a scanning electron microscope (SEM-Leica Cambridge S360).

2.2.3 CELL SEEDING/CULTURING ONTO SCAFFOLDS

Osteoblasts cells were harvested before reaching confluence from 75 cm^2 culture flasks using trypsin-EDTA (Invitrogen, Portugal), re-suspended in 5 ml DMEM culture medium and centrifuged. A cell suspension was prepared in order to seed 5 x 10^5 cells per sample. The scaffolds were placed in 48 well non-adherents culture plates. For each different formulation 6 samples were used and 300 µl of cell suspension was seeded onto the surface of each scaffold (Figure 2-9). The culture plate was transferred to the incubator for 3 hours at 37 ^oC in an atmosphere containing 5 % of CO_2. After this time, 1 ml of DMEM culture medium was added into each well and the samples cultured for different periods of time (3, 7 and 14 days). The culture medium was changed every two days.

For DNA and ALP analysis, the end of each culturing period, a batch of the scaffolds were washed twice with PBS and transferred to 15 ml tubes containing 1 ml of ultrapure water. Afterwards the samples were placed in a water bath at 37 ^oC for 1 hour (to enable the lyse of the cells). Finally, the tubes/scaffolds were frozen at – 80 ^oC until testing. The remaining scaffolds were further treated for SEM observations.

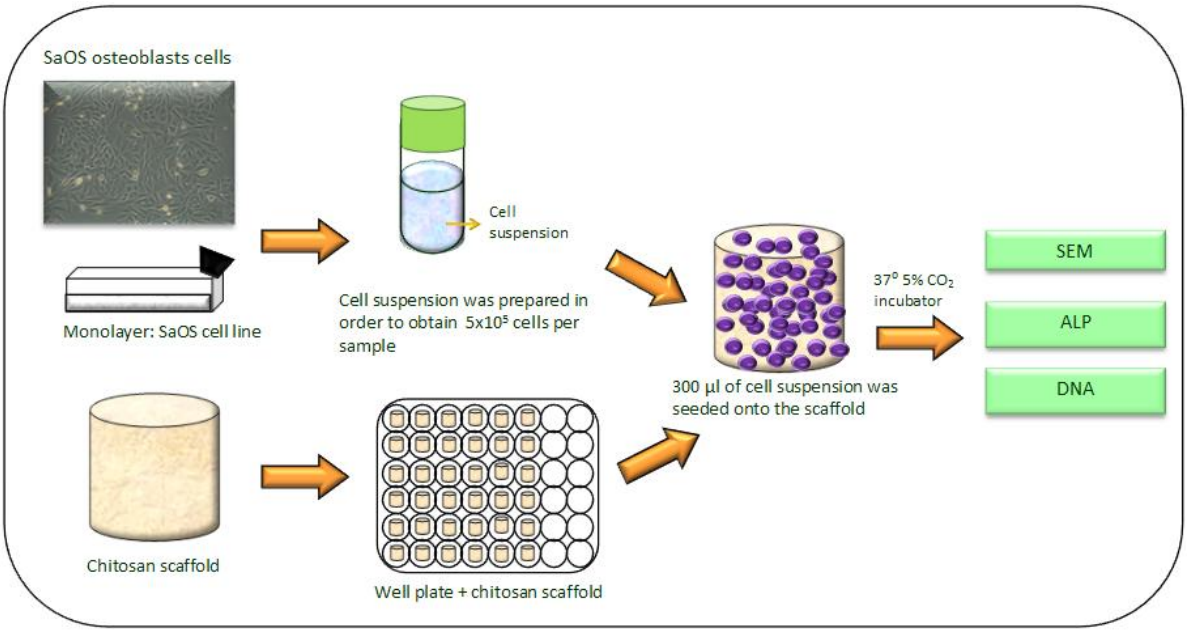

Figure 2-9. Graphical representation of the procedure used for cell seeding onto chitosan scaffolds

2.2.3.1 DNA quantification in lysed cells

The DNA content of each scaffold was measured using a Picogreen® dsDNA Quantitation Kit (Invitrogen/Molecular Probes) [34]. PicoGreen® dsDNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantify double-stranded DNA (dsDNA) in solution [35].

For the DNA quantification in scaffolds, the samples were defrosted using ultrasound bath for about 15 minutes. Afterwards, 28.7 µl of sample or standard + 71.3 µl of PicoGreen solution and 100 µl 1X TE (n = 4) were mixture using 96 culture plates (white opaque). The standard solutions were prepared according to the concentrations defined in Table 2-5. Then, the culture plates were incubated in the dark for 10 minutes. The fluorescence was read in the microplate reader at 485/20nm for excitation and 528/20 nm for emission. DNA concentration was read from standard graph. Table 2-5 showed the standard solution preparation.

The DNA quantification of each scaffold was calculated by correlation with the DNA of a known amount osteoblasts (SaOS) [36].

Table 2-5. Preparation of standard solutions - DNA assays

Concentration	Solution
0.0	1000 µl ultra pure water
0.2	2 µl standard + 1 ml ultra pure water
0.3	3 µl standard + 1 ml ultra pure water
0.5	5 µl standard + 995 µl ultra pure water
1.0	10 µl standard + 990 µl ultra pure water
1.5	15 µl standard + 985 µl ultra pure water
2.0	20 µl standard + 980 µl ultra pure water

2.2.3.2 Alkaline phosphatase (ALP) assay

ALP is an enzyme secreted from osteoblasts. It is thought to promote crystal formation in matrix vesicles by removing nucleation inhibitors. It is inhibited by glucocorticoids and parathyroid hormone (PTH) [37]. In the present study, ALP activity was quantified in osteoblasts cells to evaluate cell proliferation and differentiation on chitosan scaffolds, as the metabolic process of osteoblasts generally implies alkaline phosphatase activity and other marker for specific protein expression [38].

For this work, the ALP activity was measured using a Sigma Diagnostic kit [34], where alkaline phosphatase activity from de scaffolds/cells was quantified by the specific conversion of *p*-nitrophenyl phosphate (*p*NPP) to *p*-nitrophenol (*p*NP).

For the ALP quantification in scaffolds, the samples were prepared as describe in DNA assays, where the tubes/scaffolds were defrosted using ultrasound bath for about 15 minutes. Following this, from each tube it was taken 20 µl of sample solution and added to a 96 well culture plate (n = 4). Then, 60 µl of substrate solution ALP (p-nitrophenyl phosphate -*p*NPP) was mixed. The 96 well plate was incubated at 37^{o}C for 45 minutes in the dark. After this period the reaction was stopped using 80 µl of stop solution and then the standard solutions were placed in the same 96 well plate (the standard solutions were prepared according to the Table 2-6). The absorbance was determined at 405 nm using the microplate reader [16, 34]. Product concentration was read from standard graph. The results were expressed in *p*NP produced by scaffold/day of cell culture.

Table 2-6. Preparation of standard solutions - ALP assays

pNP Stock solution	Stop solution [µl]	Standard concentrations [µmol/ml]
0.0	160.0	0
0.8	159.2	5
1.6	158.4	10
2.4	157.6	15
3.2	156.8	20

2.2.3.3 SEM analysis of the cell seeding scaffolds

The chitosan scaffolds were prepared as was described in section 2.2.2.6 for SEM observations.

2.2.4 CELL VIABILITY OF ENCAPSULATED CELLS USING FLUORESCENT STAINS

Two fluorescent dyes were used to assess the cellular viability in encapsulated cells, namely: Calcein-AM (Invitrogen) and DAPI (Sigma).

Calcein-AM or acetoxymethyl ester of calcein, is a green-fluorescent dye widely used in cytotoxicity and cell viability assays, because it is easy to load and well retained in live cells [39, 40]. The Calcein-AM goes through the cell membrane of viable cells and is hydrolyzed by esterases into the cytoplasm demonstrating the cell viability. Calcein does not inhibit any cellular functions such as proliferation or chemotaxis of lymophocyte [41]. The amount of Calcein, hydrolyzed by esterases in cells is directly proportional to the number of viable cells in culture media. The esterases and phenol red in the culture medium interfere with the fluorescence measurement due to the cell culture medium is replaced by PBS. The excitation and the emission wavelengths of Calcein are 485 nm and 535 nm, respectively. An incubation of 10 to 30 minutes gives sufficient fluorescence intensity for the cell viability determination [42] (Figure 2-10).

DAPI or 4'-6-diamidino-2-phenylindole, is a blue fluorescent stain known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT (Adenine-Timine) and AU (Adenine-Uracil) [43]. DAPI as same as

Calcein-AM passes through an intact cell membrane and it may be used to stain live and fixed cells. For fluorescence microscopy, DAPI is excited with ultraviolet light. When bounded to double-stranded DNA, 358 nm is the maximum absorption and 461 nm is the maximum emission maximum (this emission is fairly broad, and appears blue/cyan) [44, 45].

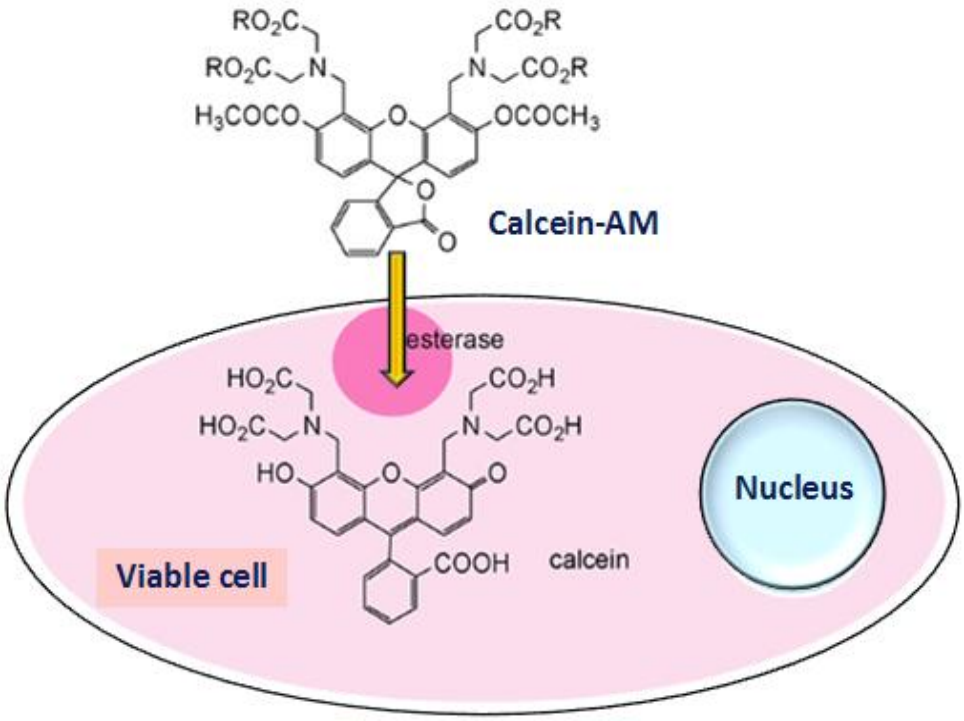

Figure 2-10. Graphical representation of cell fluorometric determination of living cell using Calcein-AM. Adapted from [41, 42].

In this work, the capsules (containing cells) were placed in an eppendorf tube containing 1 ml of DMEM and 2 µl of Calcein-AM and incubated for 20 min at 37 ^{o}C in a 5 % CO_2 incubator. Afterwards, the capsules were rinsed in DMEM culture medium, and then in a PBS solution. The PBS was replaced by 500 µl of glutaraldehyde (2.5%) for 10 minutes at room temperature to fix the cells. Then, glutaraldehyde was removed and the capsules were rinsed twice in PBS. New PBS solution (1 ml) and 2 µl of DAPI were added for 20 minuntes at 37 ^{o}C in a 5 % CO_2 incubator. Finally, the capsules were rinsed twice in PBS and observed under a fluorescence microscope (Zeiss 434301) using Alexa Flour 488 and DAPI 49 (Molecular Probes). All the procedure was performed in the dark, protecting the samples from light (Figure 2-11).

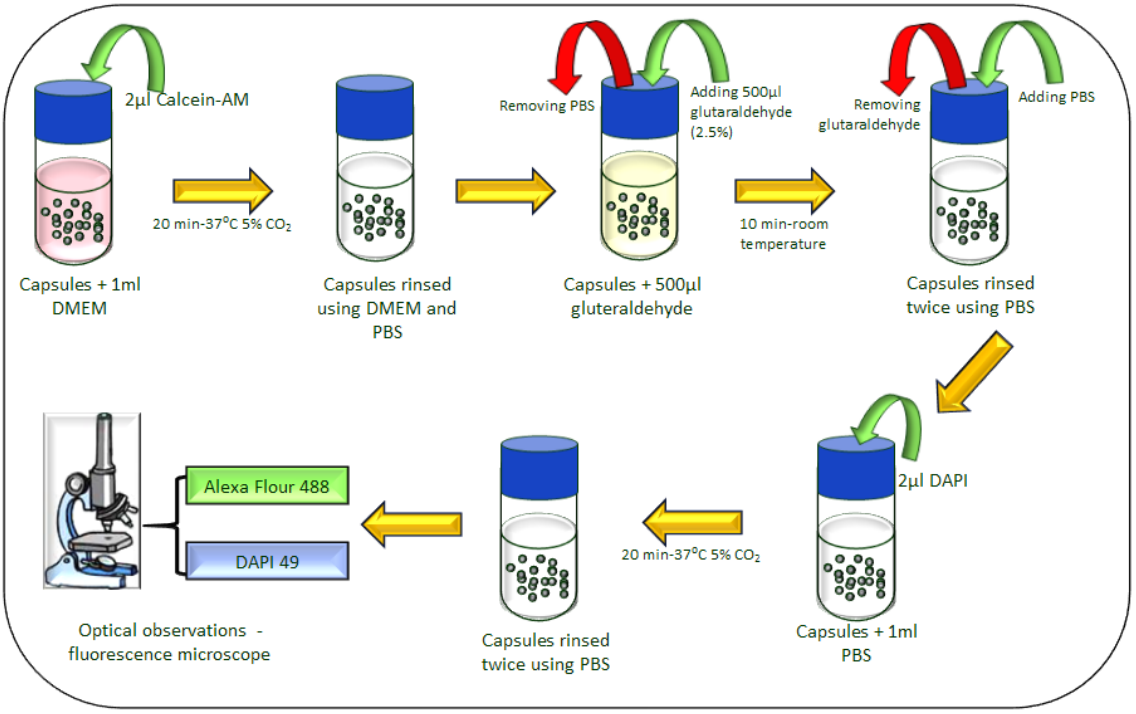

Figure 2-11. Graphical representation of the procedure used for Calcein – DAPI fluorescescent staining to assess the viability of encapsulated cells

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CHAPTER 3

3 CELL ADHESION AND PROLIFERATION ONTO CHITOSAN BASED MEMBRANES TREATED BY PLASMA SURFACE MODIFICATION [1]

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Abstract

Surface properties play a vital role in the functioning of a biomaterial. Cellular adherence and growth can be enhanced in biomaterials thought modifications of their surface. In this study it was evaluated the cellular response onto chitosan membranes surface modified by nitrogen and argon-plasma treatments. Chitosan is a natural origin polymer that exhibits suitable properties for biomedical applications such as biocompatibility and degradability. However, this polymer has shown smaller tendency for cell attachment and proliferation, which is critical in many applications. Therefore it is very important to develop techniques such as surface modifications treatments that have the ability to enhance cell adherence. In this work it was assessed the biological behaviour of chitosan membranes surface modified by argon and nitrogen-plasma treatments performed under different conditions.

Possible cytotoxicity effects of material's leachables were assessed using MEM and MTT assays. Cell attachment and proliferation onto membranes surface were evaluated analyzing the morphology of the cells by scanning electron microscopy and optical microscopy imaging, and cellular viability/proliferation using the MTS test.

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L929-fibroblasts cell line was used in all *in vitro* assays. The results showed that the extracts of membranes surface modified under all tested conditions (leachables) do not induce any cytotoxic effects. In general, it was evident an higher adhesion, attachment and proliferation of the L929 fibroblasts on treated membranes as compared to the untreated chitosan membranes. Nitrogen and argon-plasma treatment improve the surface characteristics of chitosan membranes by means of enhancing the levels of oxygen and nitrogen. The results obtained clearly demonstrated that plasma treatments constitute an effective mean of improving cell adhesion onto the surface of chitosan membranes that can be relevant in the use of this biopolymer in several biomedical applications.

Key words: cell proliferation, fibroblasts, chitosan membranes, surface modification.

3.1 INTRODUCTION

The surface modification of biomaterials has been widely studied as a method for enhancing the performance of biomaterials, improve their multi-functionality, tribological, electrical, optical and mechanical properties, as well the biocompatibility [1]. In the case of polymeric materials, surface modification has show to be a useful way to obtain functional polymers by controlling their surface properties [2]. Methods such as adsorption, plasma techniques, grafting and chemical modification have been studied to perform surface treatments [1-6].

Plasma is a mixture of electrons, negatively and positively charged particles and neutral atoms and molecules. Plasma is considered as being a peculiar state of matter, more highly activated than in the solid, liquid or gas state. From this sense, the plasma state is frequently called the fourth state of matter [2]. Approximately, since the beginning of the nineties, pulse plasmas have been used to retain the chemical structure of the precursor in thin film deposition for biomedical applications [7].

Chitosan is a known natural polymer biologically renewable, biodegradable, biocompatible, non toxic, non-antigenic and bio-functional [8-11]. It is a linear

polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), commercially produced by deacetylation of chitin [9, 12]. Chitin is a natural polymer found in many arthropods like shells of marine crustaceans (crab, shrimp and shellfish), insects and cell walls of fungi. Chitosan has been widely investigated and its has been used in several biomedical applications such as wound healing [6, 13-16], drug delivery [10], cell encapsulation [17], orthopaedic/periodontal applications [18], tissue engineering [9, 19] and radiopharmaceuticals [10] in a variety of forms including powders [20], gels [21], porous structures [22-24], fibers [25, 26], films [10], membranes [27, 28], capsules, particles or spheres [10, 29-31].

Previous studies report that chitin and chitosan have intrinsic accelerating wound healing properties [32]. The history of chitin and its derivatives as wound healing accelerators began with the studies of Pruden *et al* 1957 and 1970 [16]. The most important characteristics of chitin and chitosan for their used in wound dressing tissue are related to haemostatic action, biodegradability, biocompatibility, retention of growth factors, release of glucosamine and N-acetyl glucosamine monomers and oligomers, and stimulation of cellular activities [10]. Additionally, previously studies suggest that chitin and chitosan stimulate the migration of polymorphonuclear and mononuclear cells, and accelerate the reformation of connective tissue and angiogenesis [32]. Others studies showed that chitosan promotes the migration of the production and secretion of a large repertoire of pro-inflammatory products and growth factors, in a very early phase of healing [16].

The results of a previous study performed by our group [33] have shown that surface properties such as roughness, wettability and surface chemistry on chitosan membranes were clearly modified through the plasma surface treatment. These changes on the membrane surface influenced their cellular behaviour with respect to non-modified chitosan. Therefore, in this study, we have analysed in more detail the biological response of fibroblasts seeded onto these chitosan membranes modified by plasma treatment performed under different conditions.

3.2 MATERIALS AND METHODS

3.2.1 TEST MATERIALS

The studied materials consisted of chitosan membranes obtained by solvent casting and submitted to plasma treatment as described previously [33]. Briefly, the plasma treatment was carried on a plasma reactor equipment (PlasmaPrep5, Germany), using a power of 20 w. The duration of the glow discharge treatment was varied from 10 min to 40 min, using nitrogen gas samples designated as ChtP1- ChtP4 and argon gas samples designated as ChtP5- ChtP8 (See Table 3-1). Untreated chitosan membranes were used as control and designated by Cht.

Table 3-1. Plasma conditions used on chitosan membranes

Conditions/Samples	ChtP1	ChtP2	ChtP3	ChtP4	ChtP5	ChtP6	ChtP7	ChtP8
Power [watts]	20	20	20	20	20	20	20	20
Time [min]	10	20	30	40	10	20	30	40
Gas	Nitrogen	Nitrogen	Nitrogen	Nitrogen	Argon	Argon	Argon	Argon

All the membranes submitted to plasma surface modification treatments were cut into squares of 1x1 cm^2 and sterilized by ethylene oxide prior to all the biological studies performed.

3.2.2 CYTOTOXICITY ASSAYS

The cytotoxicity of leachables of all the treated membranes was evaluated using two cell culture methods, namely MEM extraction test (72h-short term) and MTT test, both carried out after 24 h extraction period, according to protocols described in ISO/EN 10993 [34]. Latex rubber (Dermagip, WRP) was used as a positive control and standard culture plates (Costar 96 well plates) were used as negative control. For this test, extracts of the membranes (and positive control material), were obtained by immersion in culture medium for 24 hours at 37 ^{o}C, 60 rpm.

3.2.2.1 Cell culture

A cell line of mouse lung fibroblast (L929) was selected for all the biological assays, obtained from European Collection of Cell Cultures (ECC, UK). This cell line was grown as a monolayer cultured in 75 cm^2 culture flasks, using Dulbecco's Modified Eagle's Medium-low glucose (DMEM; Sigma-Aldrich, Portugal), supplemented with 10% foetal bovine serum (FBS; Biochrom AG; Germany), 1 % antibiotic/antimycotic (Invitrogen, Portugal) and sodium bicarbonate (Sigma, Portugal) at 37 ^oC in a 5 % CO_2 incubator [35]. The culture medium was changed every 2 days and cells were grown until confluence, before starting each cytotoxicity test. For the MEM extraction test, cells were seeded in 6-well plate (n = 3) at a density of approximately 4.5 x 10^4 cell/ml. In the MTT test, cells were seeded in 96-well plates (n = 6), at density of approximately 6.6 x 10^4 cell/ml.

L929 mouse fibroblasts cell line was used in this study. Furthermore, in previous studies recognized that the fibroblasts are critical in the wound healing process [32]. It is currently accepted that the fibroblasts follow inflammatory cells into sites of tissue injury and contribute to wound healing through the synthesis of structural proteins. They also facilitate wound contraction and the reorganization of the extracellular matrix [32]. This cell line is also commonly used for cytotoxicity assessments related with biomaterial applications as these cells are able to maintain their phenotypic characteristics for long periods in culture [36].

3.2.2.2 MEM extraction test (72h)

The MEM (Minimum Essential Medium) extract test is a cytotoxicity test that has as main objective evaluating qualitatively the rates of cell damage (change of cellular morphology, inhibition of cell growth, cellular lysis and confluence of monolayer).

Extraction procedure: The chitosan membranes (60 samples of 1 cm^2 each) and the positive control (latex rubber- 60 squares of 1 cm^2) were immersed in 50 ml sterile tubes containing 10 ml of culture medium (the same used for cell culture) and placed in a water bath at 37 ^oC and 60 rpm for 24 hours. After this time, the tubes were

removed from the water bath and their content (extract material) was filtered using a membrane filter of 0.45 µm (Schleicher & Schuell Microscience, Germany) and transferred to new sterile tubes.

Test culture and evaluation of MEM extraction test (72h). For this test, the extracts of the chitosan membranes treated with plasma and argon plasma were obtained by immersion for 24 hours as previously explained. The culture medium in each well was replaced by the same amount (2 ml) of extraction fluid and the response of the cells was evaluated after 72 h. Morphological characteristics were evaluated after 24, 48 and 72 hours using an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306), and compared to the negative and positive control. The observations based on the confluency of the monolayer, degree of floating cells and changes in cellular morphology were scored, according to the parameters defined in Table 3-2, and as has reported in previous studies carried out in our group [25, 35, 37, 38].

Table 3-2. Scoring for morphological changes, confluence and cell death, used in the cytotoxicity tests

Score	Confluency [%]	Floating cells [%]	Change of cellular morphology	Inhibition of cell growth [%]
0-1	100	0	No changes during test period	0-10
1-3	90-100	0-5	Slight changes, few cells affected	10-30
3-5	60-90	5-10	Mild changes some cells round/spindle shaped	30-50
5-7	30-60	10-20	Moderate changes, many cells round/spindle	50-70
7-8	0-30	>20	Severe changes, about all cells show morphological changes	70-100

After 72h testing, the cells were detached from the culture wells by means of trypsinization. The percentage of growth inhibition was determined by cell counting, using a Neubauer camera and scored after correction for the value of the negative control. Addition of these two scores results in the final cytotoxicity response index ranging form 0 to 8 (see scores on Table 3-3).

Table 3-3. Cytotoxicity indexes used to classify the reactivity of tested samples - cytotoxic response

Cytotoxic response	Reactivity	Pass/fail
0-1	None	Pass
1-3	Slight toxic	Pass
3-5	Mild toxic	Retest
5-7	Moderate toxic	Fail
7-8	Severe toxic	Fail

3.2.2.3 MTT test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-Sigma), is a yellow vital dye that is actively converted into blue formazan crystals by mitochondrial oxidation-reduction reaction [39]. The crystals formed intracellularly were dissolved by isopropanol [40]. In this study, MTT assay was used to assess the cell proliferation and cytotoxicity on the nitrogen and argon-plasma surface modification in chitosan membranes.

This assay followed the same extraction and culture procedure described previously for the MEM test. After 72 h testing, a MTT solution was prepared (1 mg MTT/ ml in DMEM without red phenol and FBS) and 50 µl of this solution was added to the culture wells. The cultures were then incubated for 4 h at 37 ^{o}C in the dark. Following this time, the MTT solution was removed and replaced by isopropanol (100 µl) for 15 minutes, in order to lyse the cells (the purple solution appeared). Finally, the optical density (OD) was measured in a microplate reader (BIO-TEK – Synergy HT) at 570 nm with a background correction of the OD at 650 nm [37]. The evaluation expresses the variation of the absorbance in function of the concentration of extracted material. The cytotoxicity of extracts was evaluated calculating the cell viability by using the following equation (adapted of Zhang *et al*. 2006):

Eq. 1 $$\textbf{Cell viability (\%)} = (OD_s/OD_{control})^*$$

Where, OD_s is the optical density obtained in the samples (cells exposed to each extract-chitosan membrane) and $OD_{control}$ is the optical density obtained in the

negative control (cells incubated with the culture medium only) [41]. The results were expressed as percentage of cell viability.

3.2.3 DIRECT CONTACT ASSAY – ASSESSMENT OF CELL ADHESION AND PROLIFERATION OF L929 CELLS SEEDED ONTO CHITOSAN MEMBRANES

The Direct Contact assay was used to evaluate the cell adhesion and cell proliferation on the surface of chitosan membranes modified by different argon and nitrogen plasma treatments. L929 fibroblast cells were initially harvested from monolayer cultures using trypsin-EDTA. A cell suspension was prepared in order to obtain a cell density 8 x 10^4 cells/cm^2 [27]. The membranes were placed in 24 well plates and 100 µl of this cell suspension was seeded onto the samples surface (1x1 cm^2). Empty wells and coverslips (Sarstedt, USA), were also seeded with the same amount of the cell suspension to be used as controls. All the seeded samples and controls were incubated at 37 ^oC in an atmosphere containing 5 % of CO_2 for 2-3 hours. After this time, 1.5 ml of DMEM culture medium was added into each culture well and the samples cultured for different periods of time, namely 3, 7 and 14 days. The culture medium was changed every two days.

The cell adhesion and proliferation was assessed qualitatively using light microscopy (after blue methylene staining), scanning electron microscope (SEM) and quantitatively using the MTS test.

3.2.3.1 SEM analysis of the membranes

After each culturing period, the chitosan membranes were removed from cultured and washed with PBS, before being dehydrated using an increased concentration series of ethanol solutions (25%, 30%, 50%, 70%, 80%, 90% and 100% - each 30 min). The membranes were then dried at room temperature and sputter coated with gold before observation in a scanning electron microscope (SEM-Leica Cambridge S360).

3.2.3.2 MTS test- cellular viability

Cellular viability and proliferation were assessed by the MTS-CellTiter 96® aqueous one cell proliferation assay (Promega, Madison, USA). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test has been described previously [42-44]. Briefly, in this test a substrate – the MTS – is bioreduced into a brown formazan product by dehydrogenase enzymes in metabolically active cells [42, 43]. In this study, MTS solution was prepared with DMEM without phenol red and FBS in a 5:1 ratio, where 300 μl of MTS solution was added to membranes/cells and incubated for 3 hours at 37 ^{o}C, in an atmosphere containing 5 % of CO_2 in the dark. After this incubation period, 100 μl of solution from each membrane was transferred to 96-well plates (in triplicate) and the absorbance was determined at 490 nm using a microplate reader (BIO-TEK – Synergy HT). The results were expressed as absorbance obtained for the samples in each culture period.

3.2.4 STATISTICAL ANALYSIS

Statistical differences in cell viability of the different samples studied were determined by Student's t-test. $p<0.05$ was considered statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 *IN VITRO* CYTOTOXICITY ASSESSMENT

3.3.1.1 Cytotoxicity effect of leachables obtained from chitosan membranes treated by argon and nitrogen plasma on L929 cells

Regarding the short-term MEM extraction test, the results showed that no cell growth inhibition occurred after 72 h of exposition to the studied membranes extracts (Table 3-4). Morphological observations using light inverted microscope showed that cells in the negative control, exhibited normal morphology and cell proliferation (Figure 3-1a). The oppositely effect was observed in the positive control, where the

cells exposed to latex extracts showed severe morphological changes and inhibition of cell proliferation (Figure 3-1b).

Table 3-4. Growth inhibition and cytotoxic response in short-term MEM extraction test after 72h testing

Material	Inhibition of cell growth [%]	Cytotoxic response	Reactivity	Pass/fail
ChtP1	0	0.00	None	Pass
ChtP2	0	0.00	None	Pass
ChtP3	0	0.00	None	Pass
ChtP4	0	0.00	None	Pass
ChtP5	0	0.11	None	Pass
ChtP6	0	0.11	None	Pass
ChtP7	0	0.00	None	Pass
ChtP8	0	0.00	None	Pass
CHT	0	0.00	None	Pass
Latex	4	7.44	Severe	Fail

The extracts obtained from plasma surface modified membranes did not induce changes in the cell morphology and cell growth in the fibroblasts exposed to these extracts (Figure 3-1c to 3-1g), revealing a normal cell morphology and proliferation patterns, similar to those of the negative control, demonstrating the non cytotoxic effect of the membranes modified under different conditions.

The results obtaining in the MTT test showed, in general, that the leachables obtained from all chitosan membranes surface modified by argon and nitrogen plasma, did not demonstrate any cytotoxic effect, confirming the results obtained in MEM extract test.

However, the membranes treated with nitrogen plasma exhibited significant differences in terms of cell viability when compared to argon plasma treated membranes. In fact, the extracts obtained from chitosan membranes surface modified by nitrogen, the percentage of cell viability oscillated between 70 and 90% (Figure 3-2), while the chitosan membranes surface modified by argon showed lower cell viability, in special the formulations ChtP6 (20w20m-Ar) and ChtP7 (20w30m-Ar), where the percentage obtained varied between 40 and 45% (Figure 3-2). Nevertheless, the cells in contact with extracts obtained from all membranes formulations did not show significant morphological changes when compared with the negative control,

demonstrating no signs of any cytotoxicity effect. These results show that these treatments do not affect the non-cytotoxic behaviour of the chitosan membranes.

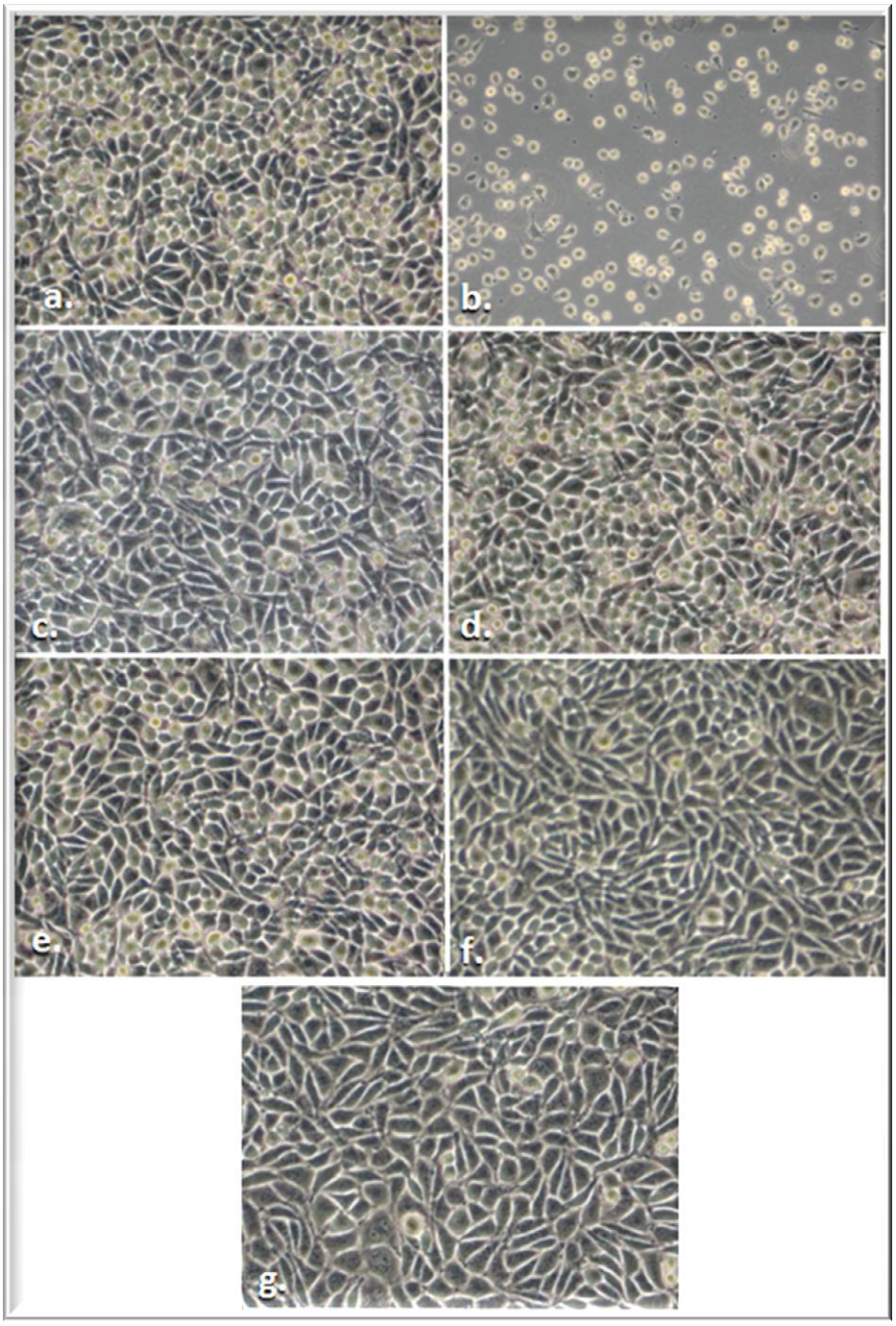

Figure 3-1. Morphology of L929 cells incubated with extracts of: a) negative control (DMEM culture medium), b) Positive control (latex extract), c) ChtP1 extract, d) ChtP5 extract, e) ChtP3 extract, f) ChtP7 extract, g) Cht extract (untreated membrane), over a 72 h period (Photographs were taken with an inverted light microscope using a 20X magnification).

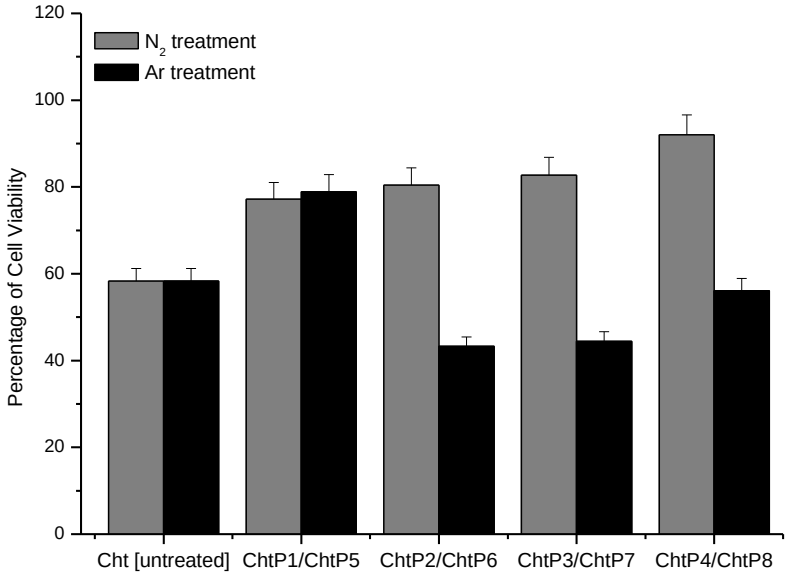

Figure 3-2. Percentage of cell viability, obtained from the MTT assay, of the fibroblast exposed to extracts of chitosan membranes surface modified by argon and nitrogen plasma (at different conditions).

3.3.1.2 Effect of surface modification in chitosan membranes using argon and nitrogen plasma treatments in cell adhesion and proliferation

Direct contact assay was used to evaluate the cell adhesion and proliferation on chitosan membranes surface modified by argon and nitrogen plasma treatments. In general, the SEM and optical microscopy observations performed after different culture periods, revealed a higher cell adhesion and proliferation of the fibroblast in contact with the chitosan membranes modified with argon and nitrogen plasma as compared to non modified membranes (Figures 3-3 and 3-4).

The cell proliferation in all chitosan membranes modified by both treatments increased with culture time, while for non treated membranes this was not evident. In fact, surface treated membrane exhibited the formation of a cell monolayer on the surfaces about 70 - 80% confluent after 7 days and 80 -100% confluent after 14 days of culture.

In the optical and SEM pictures (Figures 3-3 and 3-4) it is possible to observe that fibroblasts spread onto the surface of the membranes exhibiting a flatted morphology

that demonstrated a good adherence to the surface. In contrast, in untreated membranes, the fibroblasts exhibited round morphology, showing low cell adherence and proliferation (Cht). Chitosan untreated membrane did not induce neither cell adherence nor proliferation onto the surface with increasing culturing times.

As mentioned before, plasma treatment offers a method of altering the surface characteristics of materials without affecting the material's physical properties [45]. Previous studies [5, 45] showed that the surface functional groups resulting after plasma treatment contained high levels of oxygen and nitrogen; both groups are known to enhance cell adhesion [45]. In our case, chitosan surface membrane was modified by plasma using nitrogen and argon plasma. The literature describes [5] that nitrogen-containing plasma are widely used to improve wettability, printability, bondability and biocompatibility of polymers surfaces. Nitrogen plasma has been used to provide surface amino binding sites for immobilization of heparin on a variety of polymers surfaces [5]. Oxygen functionalities are always incorporated in nitrogen-plasma-treated polymer surfaces. The incorporation of oxygen on polymer surfaces after and during non-oxygen plasma treatments is a common phenomenon. Free radicals that are created on a polymer surface can react with oxygen during a plasma treatment. Furthermore, free radicals that remain on a polymer surface after a plasma treatment will react with oxygen when the surface is exposed to the atmosphere [5]. The presence of free radicals and oxygen incorporated by nitrogen plasma in chitosan membranes may explain our results, which demonstrate and enhanced cell spread and proliferation after plasma treatments in chitosan membranes (Figure 3-3).

Figure 3-4, shows the fibroblasts cells attachment to chitosan membranes modified by argon plasma, where the cellular response was similar to that observed in nitrogen treated membranes. Argon plasma is known for producing bond breaking and introducing oxygen groups on the surface materials [40]; these groups can be important in the cell attachment and cell growth because the presence of oxygen-containing functional groups such as carboxyl can increase acidity of the surface. On the other hand, ester, carbonate and hydroxyl groups can increase the basicity of the surface [40]. The acidic and basic groups could be enhancing the interaction of the chitosan membranes with the basic and acid amino acids of serum protein such as fibronetic and vitronectin [40, 46].

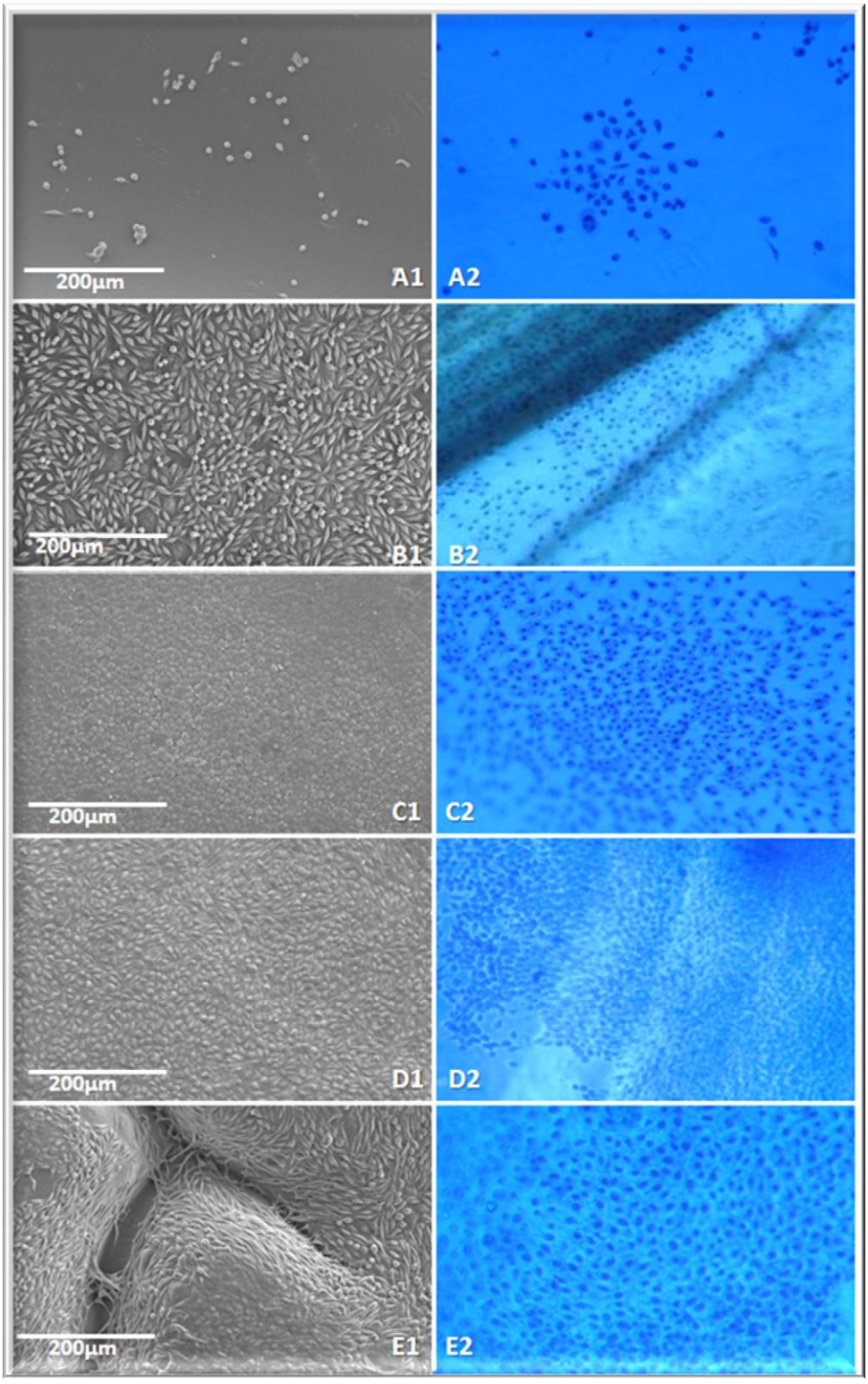

Figure 3-3. SEM (left) and optical micrographs after blue methylene staining (right-10X) of L929 fibroblasts cultured on untreated chitosan membranes (control) and chitosan membranes modified by nitrogen plasma (using different conditions) after 7 days of culture: A1-A2: Cht (untreated chitosan membranes); B1-B2: ChtP1; C1-C2: ChtP2, D1-D2: ChtP3; E1-E2: ChtP4.

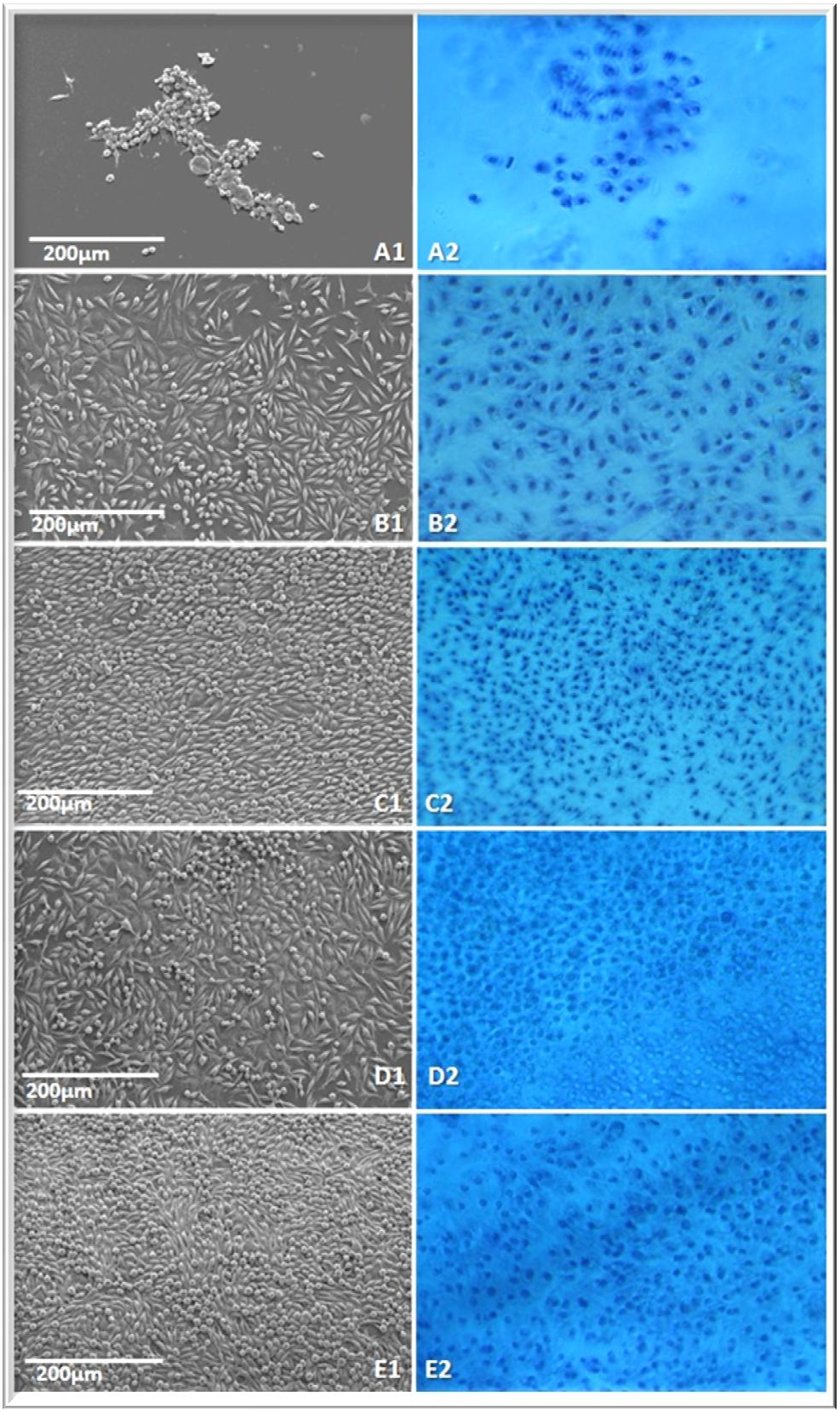

Figure 3-4. SEM (left) and optical micrographs after blue methylene staining (right-10X) of L929 fibroblasts cultured on untreated chitosan membranes (control) and chitosan membranes modified by argon plasma (using different conditions) after 7 days of culture: A1-A2: Cht (untreated chitosan membranes); B1-B2: ChtP5; C1-C2: ChtP6, D1-D2: ChtP7; E1-E2: ChtP8.

Additionally, previous studies using different plasma treatments containing high levels of oxygen and nitrogen groups exhibited same behaviour, i.e., enhanced cell adhesion was obtained [4, 40, 45], demonstrated that plasma is an efficient treatment to improve the biological response on surfaces at materials such as chitosan based membranes. Indeed, in this study, the incorporation of possible oxygen groups and free radicals onto chitosan membranes surface by means of argon and nitrogen plasma enabled high cell proliferation and cell attachment in a short period (a few days) of culture (more details about the surface modifications onto plasma-chitosan membranes can be found elsewhere [33]). In contrast, untreated chitosan membranes showed not to be favourable for cell attachment and proliferation like it has been reported in previous studies [47], probably as result of the monopolar basic nature of chitosan, which does not interact well with the bipolar extracellular matrix proteins present in the bovine serum proteins of the culture medium [40]. Moreover, the roughness modification in the chitosan membranes surface obtained after plasma treatment [33] may additionally contribute for a better adhesion of fibroblasts on these surface.

The results obtained in the MTS test (Figure 3-5) suggested that, in general, nitrogen membranes showed a higher cell proliferation as compared with argon treated membranes. Nevertheless, both treatments demonstrated enhanced cell proliferation and viability, as compared to non-treated chitosan membranes, which exhibited low cell proliferation, in accordance with the SEM observations.

The results obtained also demonstrated that the plasma conditions correspondent to ChtP2 and ChtP6 produced the best cell proliferation for all the culture periods studied. These results indicate that low power and short time conditions of exposure to nitrogen or argon plasma is enough to produce improvement in the cell growth on chitosan based membranes, where was possible to find cells with extended filipodia that have begun to spread on the chitosan surface membrane after 3 days of culture.

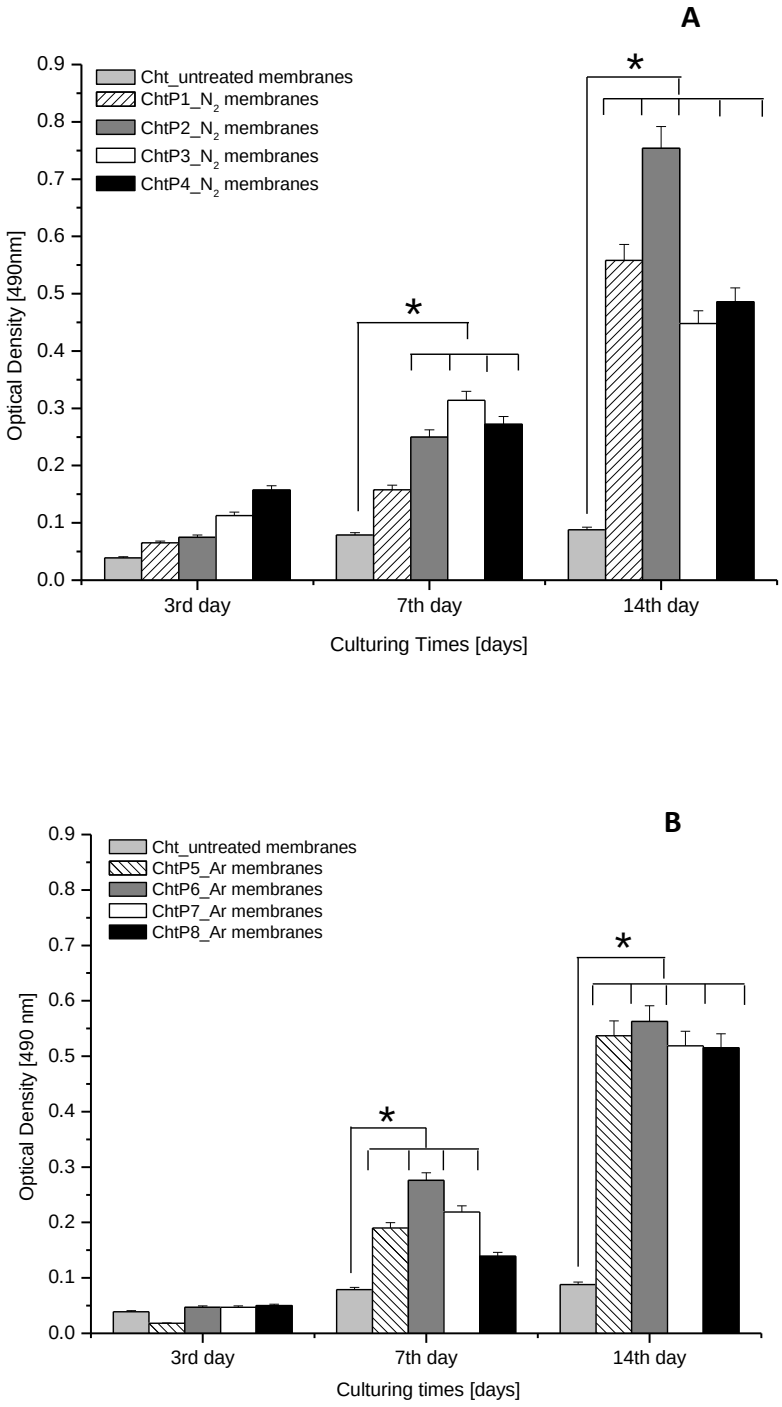

Figure 3-5. Cell viability and proliferation results obtained using the MTS test. Cell density used was 8 x 10^4 cells/cm^2. For the control, the same amount of cells was seeded on 24 culture well plates. Cells were kept in culture for 3, 7 and 14 days. A) Optical density (OD) obtained in chitosan membranes treated by nitrogen plasma, B) OD obtained in chitosan membranes treated by argon plasma.

Asterisk (*) indicates that the cell viability onto chitosan membranes treated by argon and nitrogen plasma was significantly higher ($p<0.05$) than the untreated chitosan membranes after 7 and 14 days of culture.

3.4 CONCLUSIONS

The surface modification of chitosan membranes with nitrogen and argon plasma were found to promote the proliferation of L929 fibroblasts compared to untreated chitosan membrane, where no cell adherence was observed. Cytotoxic assays showed that none of the treatments (under all studied conditions) induced any cytotoxic effect.

SEM and optical microscope observations showed a clear enhancement of cell attachment and proliferation using argon and nitrogen plasma modified membranes as compared to untreated chitosan membranes. In fact, after short culturing periods, argon and nitrogen plasma treatment seems to produce better results in terms of cells attachment and proliferation. The data obtained from the MTS test confirmed those results, where again, it was demonstrated that nitrogen and argon plasma treatment performed under optimized conditions, seems to be a suitable technique for modifying surface properties, enhancing the biological response of chitosan membranes. High levels of oxygen and nitrogen obtained after plasma treatment raise the rates of cell proliferation in comparison with untreated membranes.

In general, the present results showed that surface modification by plasma to be useful technique to treat chitosan-based material leading to enhanced cell adhesion and proliferation. These findings may be relevant in the development of chitosan-based biomaterials, including in tissue engineering applications.

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CHAPTER 4

4 DEVELOPMENT OF A NOVEL CELL ENCAPSULATION SYSTEM BASED ON NATURAL ORIGIN POLYMERS FOR TISSUE ENGINEERING APPLICATIONS[2]

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Abstract

Microencapsulated cells in biocompatible and semi-permeable polymeric membranes have shown to provide an effective system to deliver cells into a tissue/organ while protecting the host against immune response. In this work novel cell encapsulation systems were developed, based on two different natural origin polymers, carrageenan and alginate. Additionally, two different forms of carrageenan were studied, namely *iota*- and *kappa*-carrageenan, both polyanions. Chitosan was used as a polycation for the formation of the microcapsules' membranes. Furthermore, it was also studied the formation of carrageenan and alginate based microcapsules, based on an ionotropic gelation method using a sodium alginate and *iota*-carrageenan mixture extruded into an $CaCl_2$-NaCl solution. Again, chitosan was used as a polycation to coat the formed capsules. In this case, different ratios of the polymers and different pH of the chitosan solution (adjusted by adding of β-glycerophosphate) were studied in order to determine the best formulation/conditions

[2] The present chapter is based on the following paper: S.M. Luna, J.V. Araujo, M.E. Gomes, N.M. Neves, J.F. Mano, R.L. Reis. "Development of a novel cell encapsulation system based on natural origin polymers for tissue engineering applications" *Submitted to Tissue Engineering, 2007.*

to obtain the microcapsules. In addition, *iota*-carrageenan + sodium alginate capsules were also submitted (or not) to a sodium citrate treatment to analyse the differences between liquefied and non-liquefied capsules. All the developed capsules were characterized with respect to their morphology, mechanical stability and cytotoxicity. In general, it was possible to obtain microcapsules with a spherical shape from all the polymeric systems used. However, the *iota*-carrageenan + sodium alginate microcapsules exhibited the best stability and permeability, and therefore were selected for further studies, namely for preliminary cell encapsulation experiments. The obtained results also showed that the capsules produced with 30% alginate and 70% *iota*-carrageenan and a chitosan solution at pH 5.0, showed better results as compared to capsules obtained using other polymer ratios/conditions. Cell encapsulation was performed using a L929-fibroblats cell line and the *iota*-carrageenan + alginate formulation. The capsules exhibited an adequate environment for cell survival, demonstrating the high potential of the developed system for application in tissue engineering and in other cell-based therapies.

Key words**:** capsules, carrageenan, alginate, fibroblasts, cell viability.

4.1 INTRODUCTION

Encapsulation has proven to be an effective method of immunoprotecting cells or proteins for implantation into unmatched recipients allowing simultaneously for their delivery via non-invasive procedures, as capsules can be injected into a target tissue defect. In this way, cell encapsulation is a high potential approach to treat a wide range of diseases [1]. Several encapsulating techniques have been developed, which can be generally classified as micro and macroencapsulation. Microencapsulation involves a smaller cell mass or a single islet entrapped in a spherical capsule. In contrast, macroencapsulation involves large groups of cells developed in tube or disc shaped hallow devices [2, 3].

Previous studies have been demonstrated that the polymers and the procedure to develop capsules are very important for the development of successful encapsulation

systems for tissue engineering applications [4]. For this reason, several polymers have been studied for microcapsule formation, such as agarose, alginate, polyacrylates, poly (vinyl alcohol), chitosan and poly-L-lysine [5, 6], with the objective of enhancing and optimising the cell immobilization by maintaining the general cell functions and survival rates [7, 8]. In the capsules development, it is very important to obtain biocompatible and semi-permeable polymeric membranes to allow for the transport of molecules essential for cell survival within the capsules [3]. The idea of using ultrathin polymer membrane microcapsules was proposed by Chang [9] in 1964 for the immunoprotection of transplanted cells. Nearly two decades later, the concept of bioencapsulation has shown to successfully maintain glucose homeostasis in rats with diabetes using encapsulated allogenic islets [3].

The present study describes the development of microcapsules based on the combination of two natural origin polymers, namely iota-carrageenan and sodium alginate (both polyanions), as alternative systems for encapsulation of living cells. Moreover, chitosan, a well known polycation, was used for the formation of the capsule's membrane.

Carrageenan is a linear, highly flexible heteropolysaccharide with ester sulphate groups which curl, forming helical structures, with the ability to form a variety of different gels at room temperature [10]. Their main chain consists of alternating copolymers of 1,4-β and 1,3-α-D-galactopyranose and 3,6-anhydro-D-galactopyranose. This polysaccharide is extracted from red seaweed (Rhodophyceace) and exists in three major forms designated by the Greek letters: *κ* (*kappa*), *i* (*iota*) and *λ* (*lambda*). The main structural difference among these different forms of carrageenans is in the degree of substitution of the sulphate group [10, 11]. In the case of iota and kappa carrageenan, the chemical structure of iota-carrageenan differs from that of kappa-carrageenan by the presence of an additional sulphate group in the 3,6-anhydrogalactosyl residue, which confers a more hydrophilic character to the molecule [12, 13]. The differences in the textural properties of iota- and kappa carrageenan gels reflect the differences in their structures: iota carrageenan gels consist of double helices with little or no aggregation, which renders them flexible and soft. In contrast, kappa-carrageenan gels consists of aggregated helices, where the gel formed is relatively brittle and hard [13, 14].

Carrageenans are widely used in the food and other industries (cosmetic products), as thickening and stabilizing agents [15]. Other studies have also demonstrated the benefits of carrageenans in health applications, and considered this polymer to be a useful microbicide that inhibits the human immunodeficiency virus (HIV-1) infection of epithelial at nanomolar concentrations [16]. The microbicide behaviour was supported by recent studies, where it was demonstrated that carrageenans are potent inhibitor of the infectivity of genital by human papilloma virus (HPV) and pseudovirus (PsV) *in vitro* [17]. However, the use of carrageenans as polymers in the encapsulation field is reduced. Previous investigations have reported the development of capsules using carrageenan and oligochitosan polymer [10], but regarding cell encapsulation the existing studies only reported the use of carrageenan (in special *k*-carrageenan) for the encapsulation of microbial cells [18-20].

Alginate, on the contrary is the most commonly employed polysaccharide for cell encapsulation. It is composed of mannuronic (M) and guluronic (G) acids [21], extracted mainly from brown marine algae (Phaeophyceae) and as extracellular mucilages secreted by certain species of bacteria (Azobacter Vinelandii) [22, 23]. This polymer allows to develop a simple and rapid gelation with divalent metal ions such as Ca^{2+}, through binding of consecutives blocks of G-molecules on individual or different molecules [2, 24, 25]. Therefore, many researchers used alginate as the matrix to prepare microcapsules to be used as an injectable cell delivery vehicle and cell immobilization matrix [7], involving different cell sources such as myoblasts [21, 26], epithelial cells [26] hybridoma cells [27, 28], fibroblasts [29, 30], pancreatic islets [3, 30] and microbial cells [31, 32]. Additionally, alginate is relatively biocompatible and approved by the food and drug Administration (FDA) for human use as wound dressing material [33]. Nevertheless, capsules based on alginate present usually a poor mechanical stability [24, 34, 35]; furthermore, they require a further treatment with citrate to liquefied the core of the capsules and therefore enable cell survival, which further contributes to this low stability [21, 36].

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), commercially produced by deacetylation of chitin [37]. Chitin is a natural polymer found in many arthropods as shells of marine crustaceans (crab, shrimp and

shellfish), insects and cell walls of fungi and is used to produce a variety of forms including powders, gels, porous structures, fibres, membranes and films for biomedical applications [38-42]. Furthermore chitosan formulations allow a wide range of molecules to be attached, enabling its use for the delivery of growing factors [38, 43].

The main aim of this work was to combine advantageous features of alginate as a polymer for microcapsules formation with those of carrageenan, selecting chitosan as an appropriate polymer for the capsules membranes. In fact, chitosan is a polymer with positive charges it can interact with alginate and carrageenan, two anionics polymers, to create a membrane at the capsules surface [44], which could improve the mechanical stability of the structures.

This work focuses on the development of novel microcapsule for cell encapsulation systems based on two methods: polyelectrolyte complex and ionotropic gelation methods. For the first case, *iota* or *kappa*-carrageenan were used as the polyanion component, forming the capsule core, that was stabilized through complexation with chitosan in the surface. In the case of ionotropic gelation a mixture of iota-carrageenan and sodium alginate were used to obtain the capsule core by ionic cross-linking with Ca^{2+}; chitosan was used again to form the capsule membrane. In this work, iota-carrageenan was chosen as the main polymer for ionotropic gelation method for core microcapsule due to the properties of the gels that are formed, being typically soft and elastic [10, 12-14].

The obtained microcapsules were characterized with respect to their morphology by light microscopy. Mechanical stability tests allowed to select the best formulations/conditions to obtain the microcapsules used in cell encapsulation experiments. The L929-fibroblasts cell line was used as a model cell type for encapsulation studies. The cell encapsulation was performed in solid and liquefied capsules developed with alginate + *iota*-carrageenan mixture by ionotropic gelation method.

4.2 MATERIALS AND METHODS

4.2.1 MICROCAPSULES DEVELOPMENT

4.2.1.1 Materials used

Iota-carrageenan (22045), *kappa*-carrageenan (22048), sodium alginate (71238), β-glycerophosphate disodium salt (β-GP/G9891), Phosphate Buffer Saline (PBS-P4417), sodium citrate (S1804) and DAPI (4,6-diamidino-2-phenylindole/D9564) were purchased from Sigma (Portugal). Low molecular weight chitosan powder was obtained from Aldrich/448869 (Portugal). Calcein-AM (acetoxymethyl ester of calcein) was purchased from Invitrogen/C3099 (Portugal).

4.2.1.2 Adjustment of the pH of the chitosan solution using β-glycerophosphate

In this study, chitosan solutions, with different pHs were used in order to form different types of capsules by polyelectrolyte complex and ionotropic gelation method [4]. It is well known that the changes in the pH produce alterations in the protonation degree and bonding interactions in chitosan [45]. In both methodologies used for the development of microcapsules, chitosan solution was used as polycation to obtain the membrane of the capsule at two different pHs: pH 5.0 and pH 6.0.

Previous studies have demonstrated that chitosan can be neutralised up to physiological pH (7.2) using β-glycerophosphate (β-GP), without promoting the precipitation of the polysaccharide, which revealed to be a proper buffer [23, 45]. In our case, a highly diluted chitosan solution was used, preventing the formation of a thermogel. A curve of the variation of the pH in chitosan solution as function of β-GP concentration was drawn to determine the correct amount of β-GP to be added to reach the desired pH. For this purpose, a solution containing 0.0266 g/ml of β-GP was slowly added to a chitosan solution (0.30 % in 0.017 M HCl) until physiological pH. At this point (pH = 7), it was observed precipitation. The obtained curve is presented in Figure 4-1. From the curve it is evident that low concentration of β-GP is enough to reach the pHs used in this study (5.0 and 6.0).

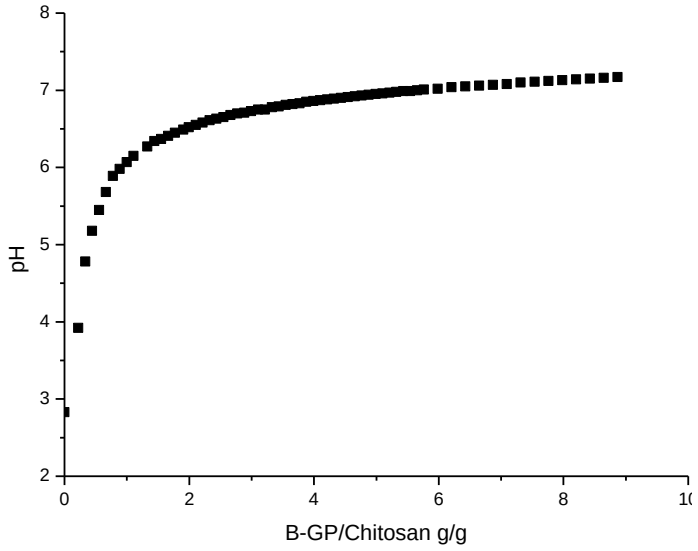

Figure 4-1. Variation of the pH relative to amount of β-GP with respect to chitosan (weight β-GP/weight chitosan g/g), at room temperature

4.2.1.3 Development of microcapsules based on *iota* and *kappa*-carrageenan

Capsules were obtained using chitosan solutions at two different pHs (5.0 and 6.0) previously adjusted with β-Glycerophosphate (β-GP) [23], as described in previous section. The microcapsules based on *iota* and *kappa*-carrageenan were developed from a pair of oppositely charged polysaccharides. For this purpose, 1 ml of an 1.5 % aqueous solution of *iota* (or *kappa*)-carrageenan was added drop by drop through a 0.3 mm needle (BD-Microfine U-100 insulin, Portugal), using a syringe pump (AL-1000, Aladdin Programmable Syringe Pump) into 20 ml of an 0.15 % chitosan in a solution at 0.0085 M HCl.

The resulting capsules were allowed to harden for 45 min at 37 ^{o}C in a water bath, washed with 0.9 % NaCl [32, 33] and immersed in a 0.05 % *iota*-carrageenan solution for 5 minutes in order to link the remaining residues of chitosan. Finally, the capsules were rinsed twice with PBS and stored at 4 ^{o}C in the same buffer.

4.2.1.4 Development of sodium alginate + *iota*- carrageenan microcapsules

Microcapsules based on *iota*-carrageenan (*i*-carrageenan) and alginate were developed by an ionotropic gelation method that was originally developed for the preparation of alginate capsules [46]. The procedure starts with the preparation of an

aqueous alginate/*i*-carrageenan mixture composed of an 1.5 % aqueous solution of each polymer, at two different ratios, namely 50/50 and 30/70 (v/v), in order to determine the best formulation. Then, 1 ml of this mixture was extruded through the 0.3 mm needle, using a syringe pump, into 20 ml of a solution composed of 0.05 M $CaCl_2$ and 0.2 M NaCl. All solutions were prepared in distilled water. The resulting microcapsules were allowed to harden for 30 min at 37 ^{o}C. Afterwards, the capsules were immersed in 20 ml of chitosan solutions (pH 5.0 and 6.0) prepared as described before for 20 min at 37 ^{o}C. Then, the capsules were rinsed with PBS for 1 min and immersed in a 0.05% *iota*-carrageenan for 5 minutes. Microcapsules with liquefied/depolymerised core were also prepared using a sodium citrate (a calcium sequestering agent) treatment. This treatment consisted in the immersion of the obtained *iota*- carrageenan + alginate microcapsules in a 0.055 M solution of sodium citrate for 1 min [7, 26]. Different times of immersion in the sodium citrate solution were used, but it was found that higher immersion times (2 or 1.5 minutes) resulted in the degradation and disintegration of liquefied capsules. Therefore, 1 minute was chosen to avoid considerable modifications in the membrane roughness in comparison with solid capsules. Finally, the obtained capsules (solid and liquefied) were rinsed twice with PBS buffer and stored at 4 ^{o}C in the same buffer for posterior characterization. The capsules were immersed in DMEM to assess the stability of the microcapsules in culture medium and also to observe the permeability into the capsules.

4.2.2 MORPHOLOGICAL CHARACTERIZATION OF THE DEVELOPED MICROCAPSULES

The size and shape of the obtained microcapsules were examined under a stereomicroscope (Zeiss – Stemi 2000-C KL 1500 LCD, 459315) and with an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306). The capsules were stained in 0.1 % (w/v) eosin dye (eosin Y solution, alcoholic with phloxine B 0.1% (w/v)/HT110316, Sigma-Aldrich, Portugal) to observe the membranes. The eosin dye was selected due to its anionic character and ability of specific binding to the amino groups of chitosan [47].

4.2.3 EVALUATION OF MECHANICAL STABILITY

Both solid and partially liquefied microcapsules produced using a sodium alginate and iota-carrageenan mixture and a chitosan solution at pH 5.0, were submitted to a short-term stability evaluation assay, in order to select the capsules presenting the best stability properties for the cell encapsulation experiments. The mechanical stability assay procedure was similar to the described by G. Orive *et al*, 2001. Briefly, 50 capsules of each type were placed in 24-well-plate (10 per well) with 800 µl of phosphate buffer saline solution (PBS-Sigma, Portugal) in each well. The culture plate was then agitated at 200 rpm and 25 ^{o}C for different periods of time, namely 1, 3, 6, 24 and 48 hours. The capsules that ruptured were counted under a stereomicroscope. Results are presented as the percentage of rupture capsules as a function of time.

4.2.4 ASSESSMENT *IN VITRO* CYTOTOXICITY OF THE DEVELOPED CAPSULES

In order to evaluate the possible toxic effects of leachables from the developed microcapsules and polyanions used, it was used the MTT Test. Latex rubber (Dermagip, WRP) was used as a positive control and standard culture plates (Costar 96 well plates) were used as a negative control. For this test, extracts of the capsules (and positive control), were obtained by immersion in culture medium for 24 hours at 37 ^{o}C and 60 rpm in a thermostatic bath.

4.2.4.1 Cell culture

A mouse lung fibroblast cell line (L929) obtained from European Collection of Cell Cultures (ECC, UK) was selected for the cytotoxicity assays. Fibroblasts are commonly used for cytotoxicity assessments related to biomaterial applications, as they are able to maintain their phenotypic characteristics for long periods in culture [48-52].

Cells were grown as monolayer in 75 cm^2 culture flasks, using Dulbecco's Modified Eagle's Medium-low glucose (DMEM-Sigma-Aldrich, Portugal), supplemented with 10% Foetal Bovine Serum (FBS, Invitrogen, Portugal), 1% antibiotic/antimycotic (Invitrogen, Portugal) and sodium bicarbonate (Sigma, Portugal) at 37 ^{o}C in a 5 % CO_2 incubator. Cells were harvested before reaching confluence from 75 cm^2 culture flasks using trypsin-EDTA (Invitrogen, Portugal) and cell suspension was prepared in order to

obtain a cell density of 6.6 x 10^4 cell/ml. Cell suspension (200 µl/well) was cultured in a 96-well culture plates in Dulbecco's Modified Eagle's Medium (DMEM). After the cells were attached to the culture plate, the culture medium was replaced by 150 µl of extracted capsules prepared in complete culture medium and incubated for 24, 48 and 72 h at 37 ^oC.

4.2.4.2 Preparation of the extracts

The extracts were obtained by immersion of 100 microcapsules (of each type) in a sterile tube containing 5 ml of DMEM and placed in a water bath at 37 ^oC and 60 rpm for 24 hours. After this time, the extract was filtered using a 0.45 µm syringe filter (Schleicher & Schuell Microscience, Germany). In the case of the positive control material, 60 squares of 1 cm^2 aproximately of latex rubber were immersed in DMEM culture medium following the same procedure described above for the test materials.

4.2.4.3 MTT test

This colorimetric assay is based on the ability of mitochondrial dehydrogenasa enzymes of living cells to convert 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) into an insoluble formazan [53]. In this assay, 50 µl of the MTT solution (1 mg MTT/ml DMEM without phenol red) was added to the cell monolayer, previously exposed to extracts obtained from the capsules, and incubated at 37 ^oC in a 5% CO_2 incubator for 4 hours. Afterwards, the MTT solution was removed and replaced by 100 µl of isopropanol in order to lyse the cells [54]. The solution became purple and the optical density (OD) was measure in a microplate reader (BIO-TEK – Synergy HT) at 570 nm, with a background correction of the OD at 650 nm. The evaluation expresses the variation of the absorbance in function of the concentration of extracted material [36, 55]. The cytotoxicity of extracts was evaluated, calculating the cell viability by using the following equation:

Eq. 4-1. **Cell viability (%)** = $(OD_{sample}/OD_{control})*100$

Where, OD_{sample} is the optical density obtained in the cells exposed to each extract/capsules and $OD_{control}$ is the optical density obtained in the cells incubated with the culture medium only (negative control) [56]. The results were expressed as percentage of cell viability.

4.2.5 CELL ENCAPSULATION

Immobilisation of the cells was performed using a L929 fibroblasts cell line in sodium alginate + *iota*-carrageenan capsules (solid and liquefied beads). Microcapsules and cell encapsulation procedures were carried out at room temperature under sterile conditions, using a laminar flow cabinet. All solutions were prepared in distilled water and filtered using a membrane filter of 0.2 µm (Schleicher & Schuell Microscience, Germany). The sodium alginate, *iota*-carrageenan and chitosan solutions were autoclaved prior to these experiments. The fibroblasts cells were initially harvested from monolayer using trypsin-EDTA (Invitrogen, Portugal), and a cell suspension containing a know amount of cells was prepared. The cells ($5x10^3$ cells per ml of the polymeric solution) were then mixed with the sodium alginate + *iota*-carrageenan solution (30/70). The cells-polymeric solution suspension was extruded into a calcium chloride-sodium chloride ($CaCl_2$-NaCl) solution, as described above using a syringe pump. The resulting microcapsules/cells were maintained in $CaCl_2$-NaCl for 30 min at 37 ^oC in a 5 % CO_2 incubator. Afterwards, the capsules containing the cells were immersed in 20 ml of chitosan solution (adjusted to pH 5.0 with β-GP) for 10 min and at 37 ^oC in a 5 % CO_2 incubator. After this, the capsules were rinsed with PBS for 1 min and immersed in 0.05% *iota*-carrageenan for 5 minutes. To obtain the liquefied capsules, the microcapsules with cells were treated with 0.055 M of sodium citrate for 1 minute. Finally, microcapsules with cells were washed twice with PBS, transferred to several wells of a 6-well culture plate, and immersed in culture medium. The encapsulated cells were cultured in a 5 % CO_2 incubator for different periods of time, namely 1, 2, 3 and 7 days. The culture medium was carefully changed every 2 days.

4.2.6 MORPHOLOGICAL CHARACTERIZATION IN ENCAPSULATED CELLS

The size and shape of the microcapsules containing the cells was analyzed using a stereomicroscope. The morphology of the cells, as well as the possible formation of cell aggregates was analyzed using an inverted light microscope (Zeiss, Axiovert 40 CFL, 459306). Additionally, viability of the cells was also assessed using the methylene blue stain.

4.2.7 ASSESSMENT OF THE VIABILITY OF ENCAPSULATED CELLS

4.2.7.1 MTS assay

Cellular viability of entrapped fibroblasts cells was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test [55, 57]. In this study, the microcapsules (30/70) containing cells (3 capsules per well) were immersed in culture medium containing MTS, in a 5:1 ratio, and incubated at 37 ^{o}C. After 3 hours of incubation, 100 µl of the solution obtained from each well (containing the encapsulated cells) were transferred to 96-well plates. The absorbance was read in a microplate reader (BIO-TEK – Synergy HT) at 490 nm. The results were expressed as absorbance obtained for samples corresponding to each culture period. As a control, was used the solution performed with alginate + *iota* carrageenan mixture (30/70) + fibroblasts cells.

4.2.7.2 Calcein-AM and DAPI fluorescents staining

Two fluorescent dyes were used to assess the cellular viability in encapsulated cells: Calcein-AM (acetoxymethyl ester of calcein, Invitrogen), is a green dye which identifies viable cells [58]; DAPI (4,6-diamidino-2-phenylindole, Sigma), is a blue dye which identifies cell nucleus in live and fixed cells [59]. In this assay, the capsules were placed in to an eppendorf tube containing 1 ml of DMEM and 2 µl of Calcein-AM and incubated for 20 min at 37 ^{o}C in a 5 % CO_2 incubator. Afterwards, the capsules were rinsed in DMEM culture medium and PBS solution. The PBS was replaced by 500 µl of glutaraldehyde (2.5%) for 10 min at room temperature to fix the cells. Then, the glutaraldehyde was removed and the capsules were rinsed twice in PBS. New PBS solution (1 ml) and 2 µl of DAPI was added for 20 min at 37 ^{o}C in a 5% CO_2 incubator.

Finally, the capsules were rinsed twice in PBS and observed under a fluorescence microscope (Zeiss 434301) using Alexa Flour 488 and DAPI 49 (Molecular Probes). All the procedure was performed in the dark.

4.2.8 STATISTICAL ANALYSIS

The results obtained in the mechanical stability tests and in the MTT (*in vitro* cytotoxicity of capsules leachables) and MTS tests (as cellular viability of encapsulated cells) are presented as means ± standard deviations. Statistical analysis of the results was performed using the Student's t-test, with a significance level of 95 % ($p < 0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 PREPARATION OF THE MICROCAPSULES

4.3.1.1 Development of microcapsules based on *iota* and *kappa*-carrageenan

The results showed that were possible to produce capsules using *iota* or *kappa*-carrageenan via complexation with low molecular weight chitosan. Observations using a stereomicroscope and inverted light microscope revealed that *kappa* and *iota*-carrageenans used in this study formed approximately spherical shape microcapsules with a diameter in the range 2.0-2.2 mm (Figure 4-2). It is well known that both carrageenans have polyelectrolytic character and form a thermoreversible gel which is induced by selected monovalent and/or divalent metal cations [10, 13].

The pH of the chitosan solution used during the capsules formation influenced the capsule structure. The capsules formed at low pH (5.0), exhibited a smoother surface than the capsules formed at pH 6.0. This is probably due to the fact that, at pH 5.0, the capsules have the high degree of protonation of the chitosan that can induce a more homogeneous complexation. These results are in accordance with alginate/oligochitosan and carrageenan/oligochitosan systems developed in other studies, where a more compact polyelectrolyte complex with higher crosslinking density was formed [10, 35, 47].

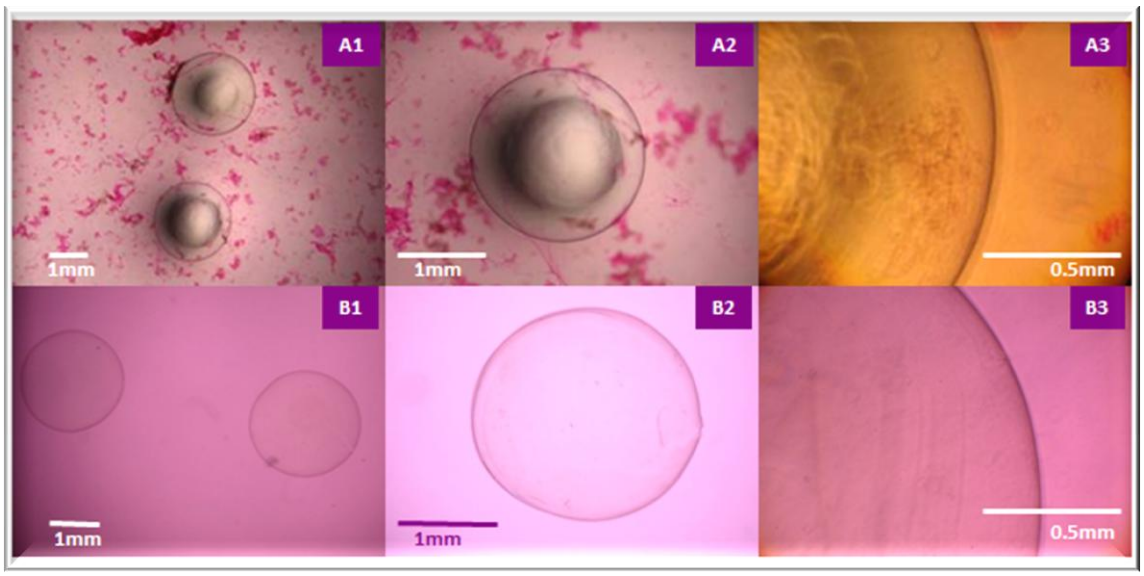

Figure 4-2. Optical microscopy images taken at pH 5.0 of the obtained microcapsules based on A1-A3) *kappa*-carrageenan and B1-B3) *iota*-carrageenan.

Previous studies have demonstrated that the thickness and strength of the capsule wall can be controlled, to some extent, by varying the molecular weight of the constituent polymers. Lower molecular weight forms diffuse through the capsule membrane more rapidly and thus produce thicker walls before being hampered by the diffusion distance. For instance, lower molecular weight chitosan produce capsule walls that are both thicker and less elastic than those made with higher molecular weight forms [4, 60, 61]. As a result, the highly diluted chitosan solution and its low concentration (0.15 %) using in the capsules developed can produce a membrane with less elasticity but with fewer incidences of physical defects. Moreover, the low Mw chitosan, used in this assay can increase its capacity to penetrate and interact with the core polymer [4].

In general, *k*-carrageenan generated thicker gels in the core as compared to *i*-carrageenan (Figure 4-2). We expect then that microcapsules based on *k*-carrageenan will prevent the exchange of gases and nutrients, harming the viability of the encapsulated cells. Therefore, *iota*-carrageenan was selected for further studies, concerning the development of microcapsules based on the combination of this polymer with alginate. Previous studies [35] have demonstrated that *iota*-carrageenan and alginate are similar because both polymers have one anionic group per glucosic unit, producing a more compact and resistant capsule in comparison with *kappa*-

carrageenan which has a lower degree of substitution (one anionic group per two glucosic unit).

4.3.1.2 Development of sodium alginate + *iota*-carrageenan microcapsules

The capsules based on alginate and *iota*-carrageenan were produced from a mixture of two "strong" polyanions extruded into a gelling agent ($CaCl_2$) and antigelling (NaCl) [62]. According to optical microscope observations, the capsules performed exhibited spherical shape, thin and smooth surface and revealed good stability in PBS and in culture medium (See Figure 4-3). The diameter of the solid capsules (non-liquefied) was about 2.0 mm, while the liquefied capsules exhibited average diameters of 2.3 mm. The increase in diameter in liquefied capsules can be explained by the citrate treatment, that can enhance the swelling of the capsules [28, 36]. The citrate, a sequestering agent, generates a strong bound between calcium ions and non-gelling sodium or potassium ions. The decrease of the Ca^{2+} bound between the alginate chains increase the swelling pressure inside the capsule, modifying the original diameter [1, 34].

On the other hand, the capsules obtained with different *iota*-carrageenan and alginate ratios and different pHs showed similar size, shape and stability in PBS and culture medium. However, the capsules obtained at pH 5.0 exhibited a smoother membrane and better handling properties in comparison with capsules obtained at pH 6.0. In fact, previous studies have demonstrated that the pH of a solution is a handy tool for modulating polyelectrolyte membranes [4]. For instance, the cationic polymer chitosan is soluble at low pH, but its solubility decreases as alkaline pH is approached. Moreover, the micropores of chitosan membrane capsule assume minimum dimensions at low pH, but at about pH 6.0 and higher, repulsive forces produce the pores to open up, and consequently, membrane permeation rise [4].

Previous studies [62] concluded that NaCl is one of the factors determining membrane permeability as well as surface topology. Sodium ions control the initial stage of complex formation. The negative charges of the reacting polymers suppress the reaction of calcium ions with the polyanion matrix. Therefore, by increasing the ratio of Na^+ to Ca^{2+} for a given polyanion mixture, it is possible to obtain a wrinkle-free surface [4].

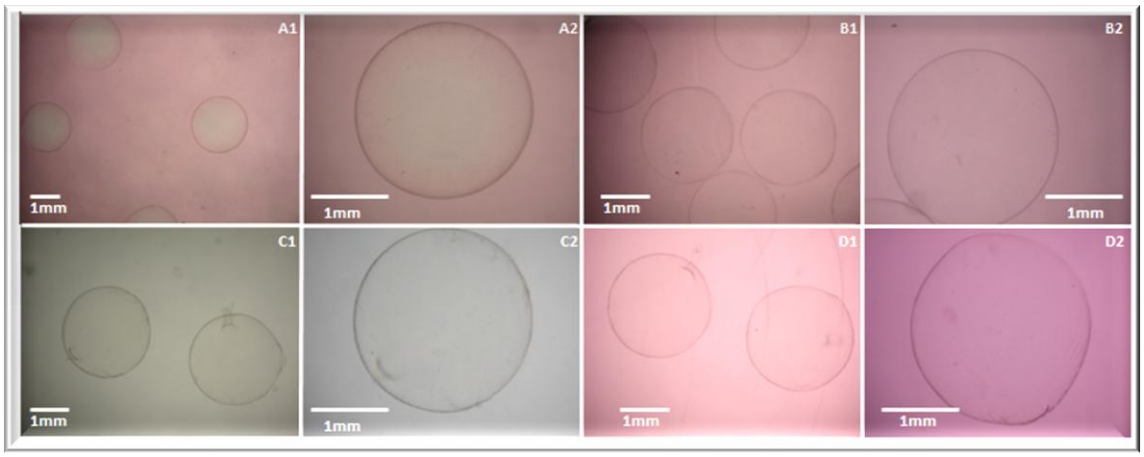

Figure 4-3. Optical microscopy images of solid capsules obtained using different polymer ratios and at different pH of the chitosan solution: A1-A2) microcapsules obtained from a mixture of sodium alginate/*iota*-carrageenan (30/70) at pH5; B1-B2) microcapsules obtained from a mixture of sodium alginate/*iota*-carrageenan (30/70) at pH6; C1-C2) microcapsules obtained from a mixture of sodium alginate/*iota*-carrageenan (50/50) at pH5; D1-D2) microcapsules obtained from a mixture of sodium alginate/*iota*-carrageenan (50/50), at pH6. The microcapsules (A, B, D) were dyed with eosin for a better observation of the chitosan membrane.

4.3.2 ASSESSMENT OF MECHANICAL STABILITY

The mechanical stability of microcapsules is one of the major concerns in the design of cell immobilisation devices for therapeutic purpose where it is necessary a good mechanical stability in order to prolong their long-term *in vivo* function [63]. In this study, a stability assay was performed to analyse four types of the capsules developed: solid capsules 30/70 (SA-IC sodium alginate + *iota*-carrageenan), liquefied capsules 30/70 SA-IC, solid capsules 50/50 SA-IC and liquefied capsules 50/50 SA-IC. The results obtained by the short-term stability showed a similar percentage of ruptured capsules for all the studied systems (Figure 4-4). Notwithstanding, capsules produced using a mixture 50/50 SA-IC of the polymers exhibited lower stability in the mechanical study (± 10 % more of ruptured capsules) as compared to capsules formed using 30/70 SA-IC polymers. These results might be explained by the increase in the *iota*-carrageenan content of this formulation as the higher amount of ionic sulphate groups that repel each other, contribute for maintaining the molecules highly extended. Furthermore, the gradual substitution of alginate, a rigid polymer, by

carrageenan, an extremely flexible polymer provides the flexibility required to adapt to the varying stress of tidal and wave motion caused by the agitation [25, 64].

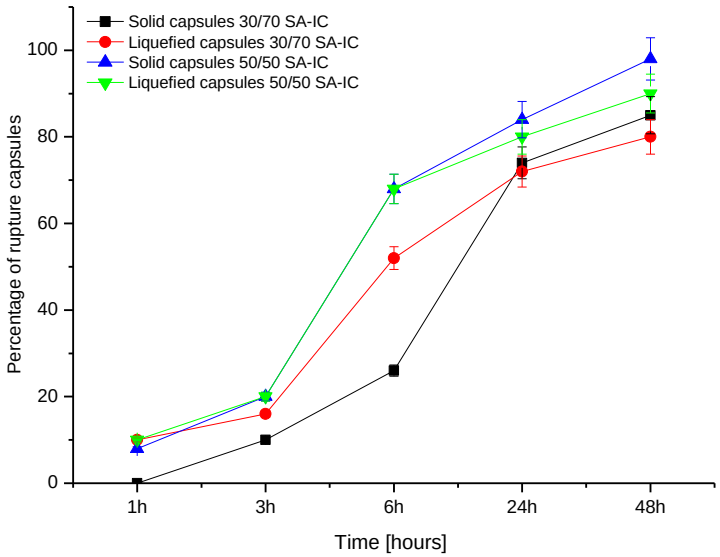

Figure 4-4. Percentage of ruptured microcapsules (correspondent to different sodium alginate/*iota*-carrageenan ratios) as a function of time.

Figure 4-4 shows the percentage of ruptured capsules. In the first six hours, liquefied and solid (non-liquefied) 50/50 SA-IC capsules, presented a similar behaviour. However, for 30/70 SA-IC composition, solid capsules presented a higher mechanical stability for that same time period. This suggests that the decreasing stability is caused by the swelling effect related to the treatment with sodium citrate, which affects more the capsules with higher concentration of alginate. After 24 h, both 30/70 and 50/50 non-liquefied capsules present an increase in the percentage of rupture that can be related to the mass transfer (in our case is related with the PBS solution transfer through of capsule membrane), which from this period may develop an important role on capsules stability. As it is well known, the mass transfer is increased in liquefied capsules, and the equilibrium is reached faster than in non-liquefied ones. Nevertheless, before reaching the equilibrium, the mass transfer in non-liquefied capsule may occur predominantly from the solution to the interior of the capsules, leading to their burst (Figure 4-5).

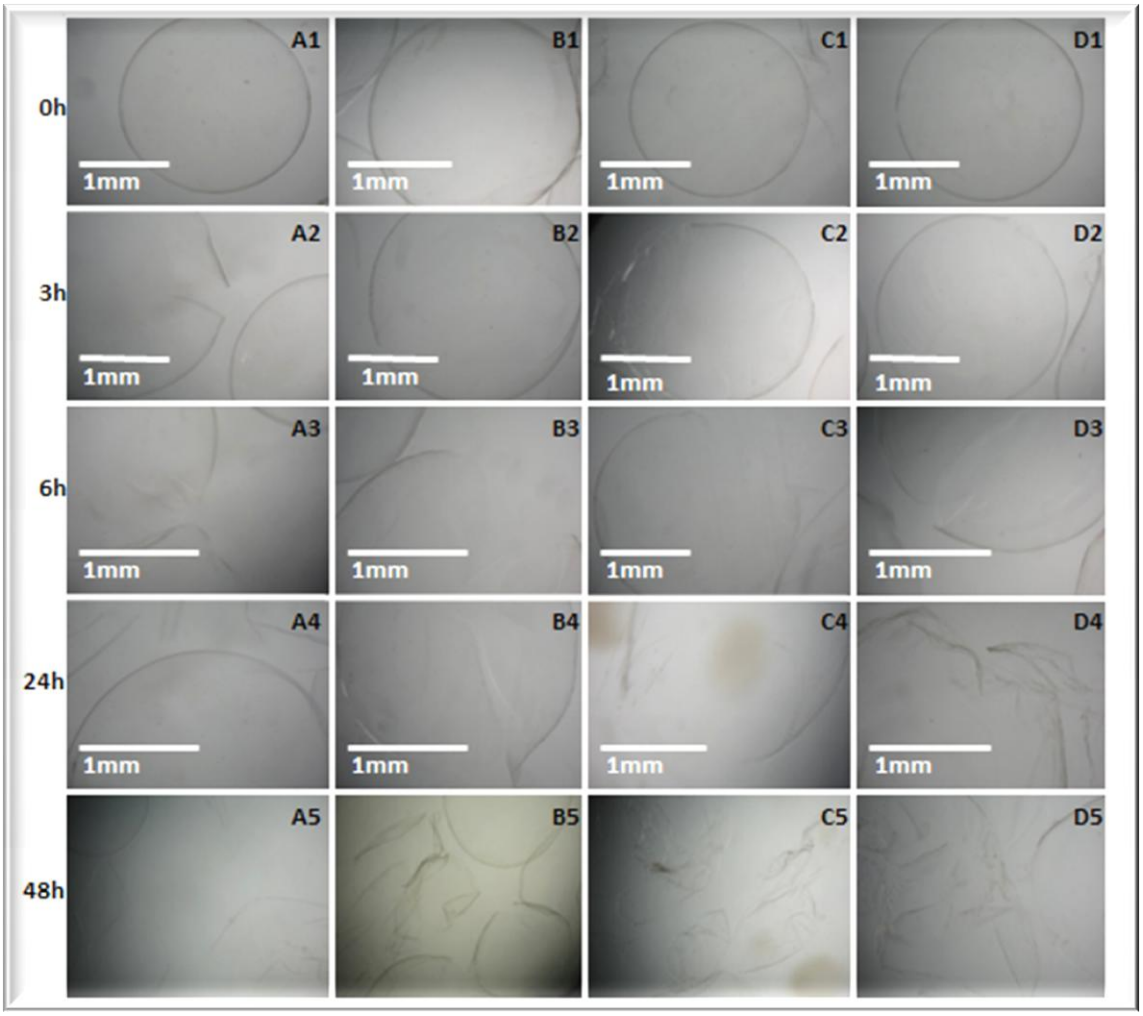

Figure 4-5. Optical microscopy images of the capsules after the short-term stability study carried out at different time periods. A) 30/70 solid capsules, B) 30/70 liquefied capsules, C) 50/50 solid capsules, D) 50/50 liquefied capsules. Photographs were taken with a stereomicroscope.

Previous studies [28, 34, 36] have reported that solid capsules (non-liquefied) were mechanically more resistant than liquefied capsules. That can be attributed to the swelling effect induced by the citrate solution during production of the liquefied capsules. Nevertheless, in this work, statistically significant differences were not found in the stability of solid and liquefied alginate/*i*-carrageenan capsules, probably as a result of the structure and distribution of the mixed polymers used in this study, during capsule formation. According to these results, the capsules 30/70 SA-IC (solid and liquefied) were selected for the posterior cell encapsulation assays.

4.3.3 *IN VITRO* CYTOTOXICITY ASSESSMENT

The metabolic activity and viability of the fibroblast cultured with leachables extracted from 30/70 and 50/50 SA-IC solid capsules were assessed using MTT test for three different periods of culture (24, 48 and 72 hours). Optical observations (before adding the MTT solution) revealed a good cell growth and attachment. Moreover, no changes in cell morphology were detected in comparison with negative control (Figure 4-6), which demonstrated the non cytotoxicity of these capsules formulations. Indeed, the morphological characteristics of fibroblasts were maintained during the three days of extract/cell culture and no cell lyse was observed.

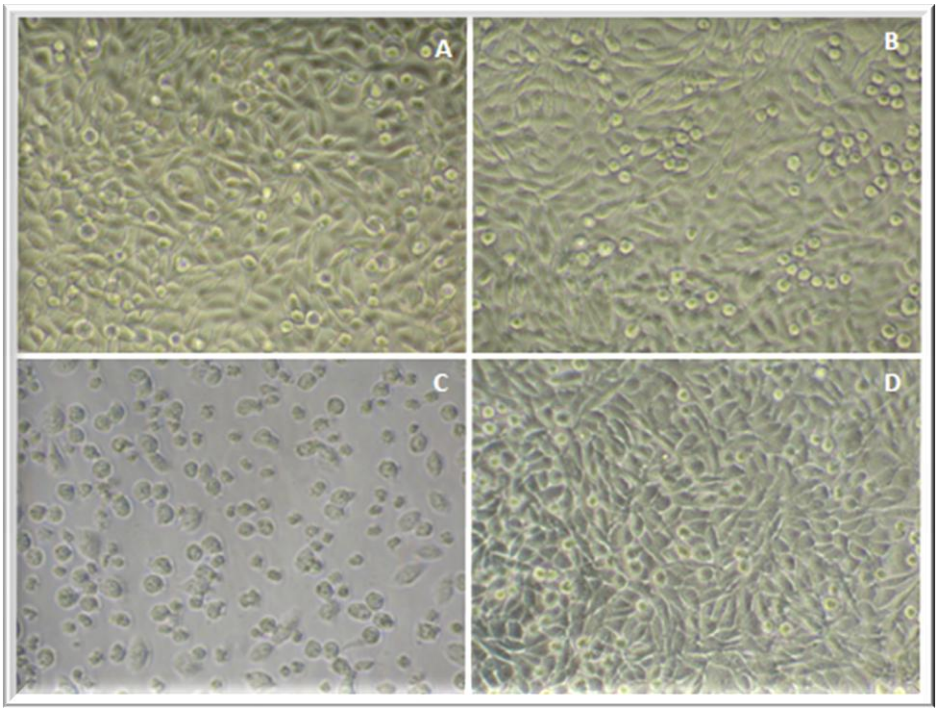

Figure 4-6. Morphology of fibroblasts exposed to: a) 30/70 SA-IC capsules extracts; b) 50/50 SA-IC capsules extracts; c) latex extracts (positive control) and d) culture medium (negative control) incubated 72 h at 37 ^{o}C. Photographs were taken with an inverted optical microscope (20X).

Nevertheless, the percentage of cell viability determined using MTT test in cells exposed to extracts of 50/50 SA-IC capsules was lower as compared to the cells exposed to the extract obtained from the 30/70 SA-IC capsules (Figure 4-7). These results might be explained based on the fact that the capsules with higher content of alginate (50/50) than 30/70 capsules could produce leachables, which induced some

reaction from the cells exposed to their extracts. Notwithstanding, in each capsules type the percentage of cell viability was very similar for all the culturing periods studied.

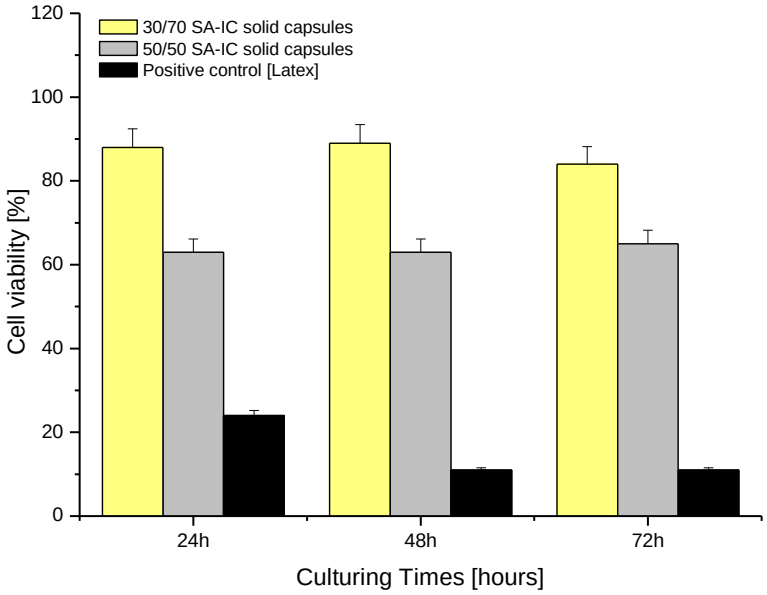

Figure 4-7. Metabolic activity of the L929 fibroblasts cultured with extracts obtained from 30/70 and 50/50 SA-IC solid capsules for 24, 48 and 72 hours, determined using the MTT test.

4.3.4 ENCAPSULATED CELL VIABILITY AND PROLIFERATION

4.3.4.1 Optical microscopy analysis

According to results obtained, mainly those regarding the mechanical stability evaluation, it was decided to use the 30/70 SA-IC solid and liquefied capsules, obtained using a chitosan solution of pH 5.0, for the cell encapsulation experiments. The results showed that in both types of capsules, the encapsulated cells maintained the viability. The optical microscopy images show the capsules in culture medium and stained with blue methylene, where the blue colour on the nucleus of the cells indicates the presence of living cells (Figure 4-8). Additionally, 7 days post-encapsulation, the cells exhibited similar shape and distribution in solid and liquefied capsules and no cell aggregates were observed. This is a relevant observation since previous studies showed that the cell aggregates could produce necrotic spaces within the capsules as a consequence of poor mass transfer [36, 53].

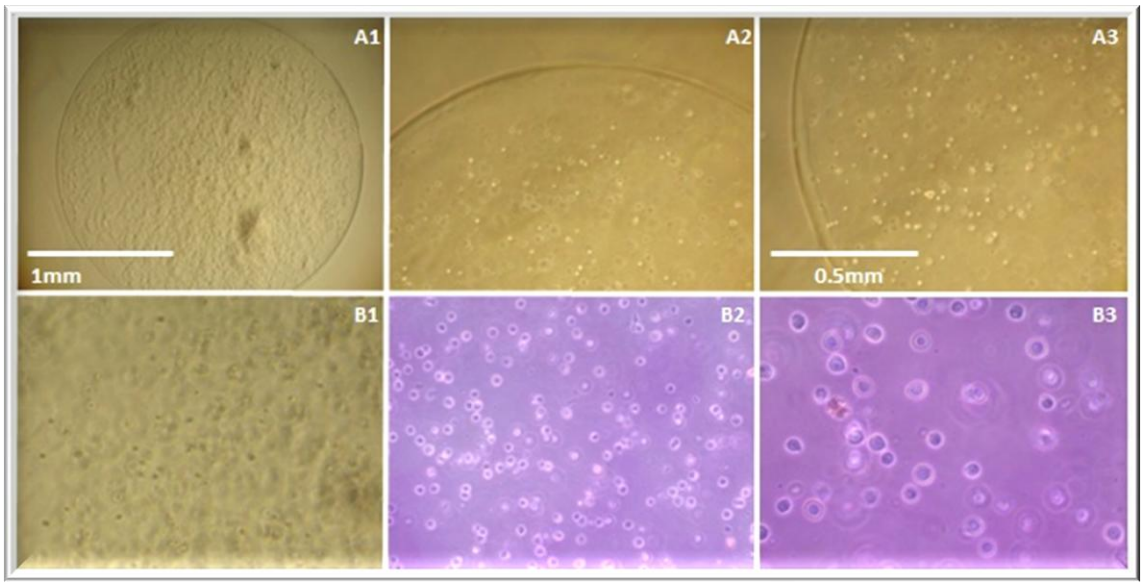

Figure 4-8. Optical microscopy pictures showing encapsulated fibroblasts (density $5x10^3$ cells per ml) within sodium alginate/*iota*-carrageenan (30/70) solid capsules pH 5.0. A1, A2 and A3) cells within solid capsules in culture medium (5X and 10X respectively); B1) cells within solid capsules in culture medium (20X); B2 and B3) Cells within solid capsules stained with blue methylene (20X and 40X respectively), after 72 h.

Previous studies [26, 36] have demonstrated that cells enclosed in liquefied beads present an improved growth profile in comparison to the cells entrapped in solid capsules. However, in the system studied in this work, the solid and liquefied capsules formed using sodium alginate/*iota*-carrageenan as polyanions showed a similar behaviour. These results can be explained by the mixture and proportion of polymers used, as *iota*-carrageenan is present in higher proportion (70%) than sodium alginate (30%). In fact, *iota*-carrageenan confers an elastic structure to the core of the capsule, providing it with higher flexibility, in opposition to sodium alginate that confers a rigid structure [64, 65].

4.3.4.2 MTS test

Cellular viability and proliferation were also determined using the MTS test after 72 hours of culture, where fibroblasts cells encapsulated into capsules 30/70 SA-IC (solid and liquefied) were exposed to MTS solution. The solution used to form these capsules (sodium alginate/*iota*-carrageenan mixture 30/70) mixture with cells, was

also evaluated in this test as a control. The results suggested that the cell viability decreased in encapsulated cells but increased in the cell/solution mixture (Figure 4-9). However, the cell viability of both types of capsules did not show significant differences after 72 h of incubation, indicating that the citrate treatment may not be relevant to improve the capsules permeability an hence for cell viability, using this polymeric formulation.

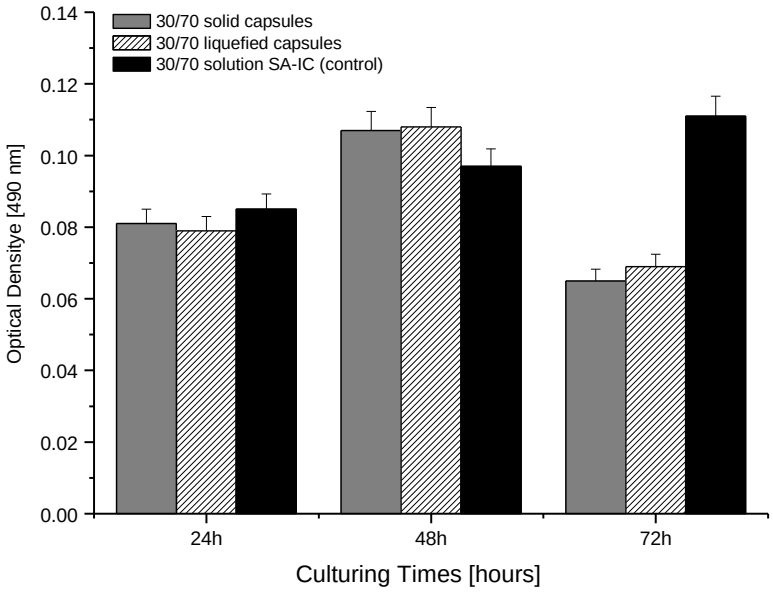

Figure 4-9. Cell viability and proliferation of fibroblast entrapped within SA (sodium alginate)/IC (*iota*-carrageenan) capsules and in the polymeric solution SA/IC (ratio: 30/70), determined using the MTS test.

On the other hand, with the culturing period, cell/solution showed increased cell viability with a homogeneous growth further demonstrating the non cytotoxicity of this mixture, confirming the results obtained in MTT assays. These results also indicate that the decrease in cell viability over time in the capsules may be due to the decrease in permeability resulting from the membrane formation during the capsule preparation. In fact, the cell growth is better in cells/solution SA-IC than in the capsules because the molecules and gases did not have to cross the membrane present in the capsule to reach the cells.

4.3.4.3 Fluorescents staining

It is known that the Calcein-AM only stains viable cells and DAPI interacts with the double DNA in viable and fixed cells [58, 59]. In this study, the results obtained using fluorescent staining confirmed the cellular viability in fibroblasts cells encapsulated into capsules 30/70 (solid and liquefied), where it was evident the green and blue colour after 7 days of cell culture demonstrating the living cells (Figure 4-10). These dyes are indicative of cell membrane integrity and permeability. Additionally, these results confirm the permeability and non toxicity of the capsule developed with sodium alginate and *iota*-carrageenan polymers. Once again, it was not possible to determine differences in cell viability between encapsulated fibroblast into solid and liquefied capsules. The cells exhibited similar distribution and same fluorescence pattern in both type of capsules. However, after 7 days, the cells in both capsules showed low fluorescence, possibly because the decreased cell viability.

In general, the results obtained are indicative that Calcein-AM and DAPI were able to penetrate alginate-carrageenan capsules contain living fibroblast in suspension with intact membrane, revealing, thus, cell viability in this structure. All capsules developed did not show differences in the cell viability and proliferation, confirming the results obtained in the MTS test. Moreover, the observations using fluorescents staining also did not demonstrate formation of cell aggregates.

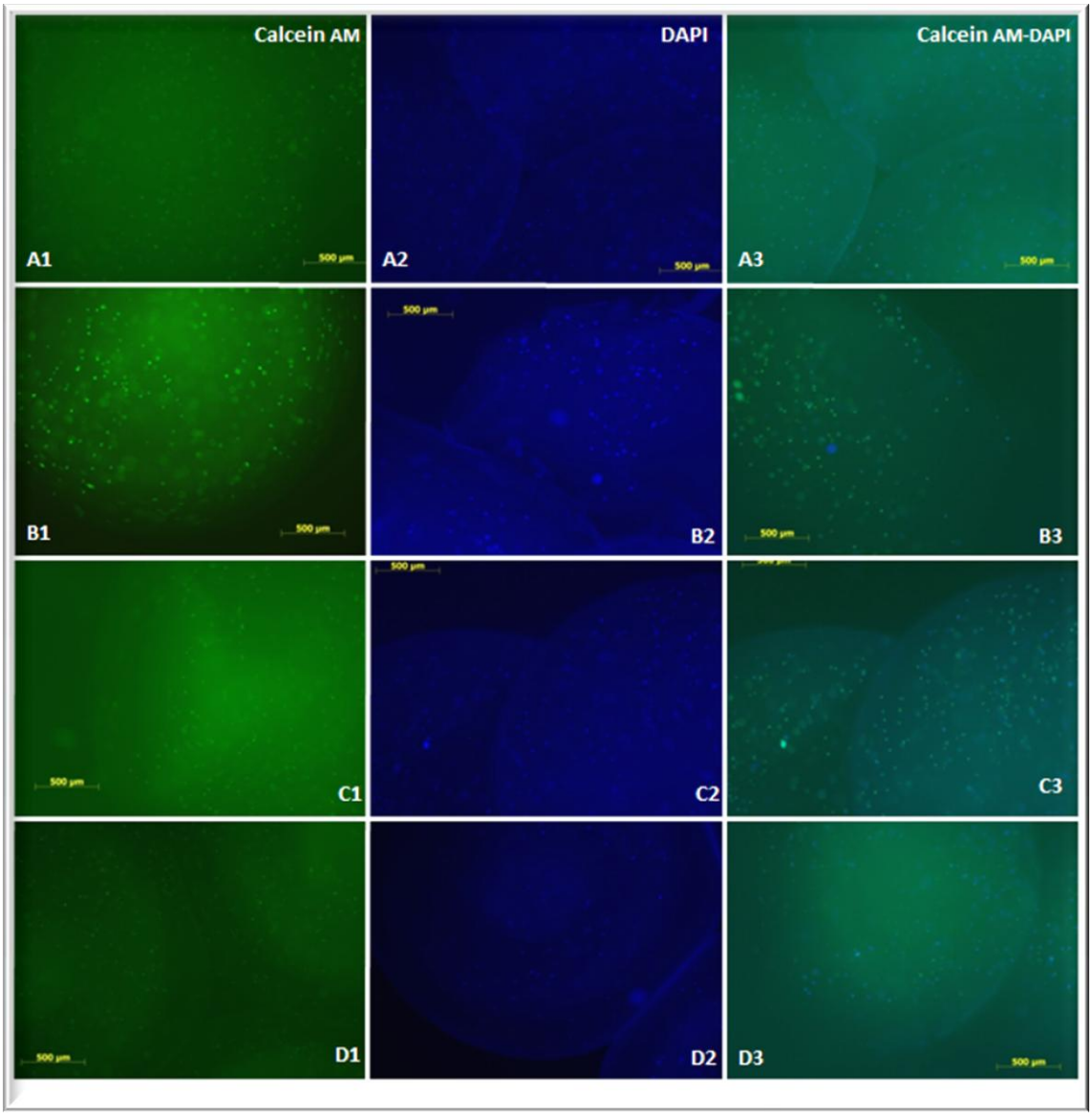

Figure 4-10. Images obtained in the fluorescent microscope of encapsulated fibroblasts stained with Calcein-AM and DAPI dyes after: (A1-A3) 24 h, (B1-B3) 48 h, (C1-C3) 72 h and (D1-D3) 7 days.

4.4 CONCLUSIONS

All the developed capsules based on *iota* and *kappa*-carrageenan as well as these made from mixture of *iota*-carrageenan with alginate, exhibited spherical shape, smooth surface and revealed good stability in PBS and in culture medium.

Iota-carrageenan capsules showed suitable properties for cell encapsulation and was selected to developed alginate/carrageenan capsules.

Microcapsules developed via ionotropic gelation method based in the mixture of sodium alginate and *iota*-carrageenan solutions showed to be suitable for the

development of stable capsules, using chitosan as the main component of the capsule's membrane. The mechanical properties of these capsules were mainly influenced by the alginate/carrageenan ratio used.

The results of short-term stability evaluation suggested that capsules with high content of *iota*-carrageenan are more stable and their stability is not negatively affected by the citrate treatment.

Cell encapsulation experiments showed that in both types of 30/70 SA-IC capsules (solid and liquefied) the cells maintained their viability and did not form cell aggregates. Moreover, these experiments demonstrated that the citrate treatment is not necessary to improve the permeability of *iota*-carrageenan/sodium alginate capsules with a high content of carrageenan (70%).

The results obtained suggest that this new polymeric system based on mixtures of alginate with *iota*-carrageenan produce adequate and promissing systems for cell encapsulation that may found several applications in tissue engineering on other cell therapies.

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CHAPTER 5

5 PRELIMINARY STUDIES OF CELL ADHESION AND PROLIFERATION OF OSTEOBLASTIC-LIKE CELLS ON CHITOSAN POROUS HYBRIDS SCAFFOLDS

5.1 INTRODUCTION

Three dimensional porous structures have been recognized as the most Three dimensional porous structures have been recognized as the most appropriate scaffold design to sustain cell adhesion and proliferation [1, 2]. The material composition as well as structural characteristics such as external and internal architecture are crucial for the successful outcome of all scaffolds-based bone tissue-engineering strategies [3-5]. These materials must comply with a large number of requirements such as biocompatibility, biodegradability, apart from adequate porosity and permeability [6, 7] in order to allow the ingress of cells and nutrients, and the appropriate surface chemistry for enhanced cell attachment and proliferation [8].

Surface characteristics such as topography, chemistry or surface energy, play an essential role in cell adhesion onto biomaterials. As a result, cell attachment, adhesion and spreading belong to the first phase of cell/material interactions and the quality of this first phase will influence the capacity of cells to grow and differentiate when contacting with the tissue to regenerate [9].

A number of fabrication technologies have been applied to process biodegradable and bioresorbable materials into 3D-polymeric scaffolds of high porosity and surface area. The conventional techniques for scaffold fabrication include fibre bonding [10, 11], solvent casting [11-15], particulate leaching [16], gas foaming [17-19], phase separation/emulsification [20-23], membrane lamination and melt molding [7, 24].

Additionally, others methods include extrusion, *in situ* polymerization [8, 25], 3D-printing and selective laser sintering [6, 26, 27]. These last engineering technologies are highly controllable, reproducible and facilitate the manufacture of well-defined 3D-structures [28].

In the case of bone applications, several scaffolds materials have been investigated including hydroxyapatite (HA) [29, 30], and natural polymers such as collagen [2, 31], fibrin [32], chitosan [33-40] chitin [7, 37] and starch based polymers [41-46], exhibited good biocompatibility and some cases osteoconductive properties.

The aim of this work was to assess the cell response to several chitosan based scaffolds formulations.

Previous studies showed that chitosan scaffolds are osteoconductive and can enhance bone formation both *in vitro* and *in vivo* [34, 36, 38, 47]. In spite, of its general acceptance as tissue biocompatible material, chitosan is mechanically weak and unstable and unable to maintain a predefined shape for transplantation as a result of swelling [36].

In our group, chitosan based scaffolds (porous structures) have been produced by freeze-drying [25, 30, 33] . The scaffolds's properties, such as porosity can be controlled by the treatments applied to the scaffolds, such as crosslinking, neutralization with alkali solutions and control of the pH of the initial polymeric solution. It is possible to control the porosity in the 20-1000 μm range [48].

Methodologies as sol-gel process focus on the modification of the chitosan structure by introducing chemical cross-linkages between the reactive groups of polymer and the siliceous skeleton. Then, a more effective bonding between the organic and the inorganic phases can be achieved, improving the mechanical performance, chemical stability and controlled water absorption [33].

In this work, it was assessed the cell response to several chitosan based scaffolds formulations that are being studied for applications as supports for bone tissue regeneration. The biological response of the scaffolds was evaluated by seeding and culturing the matrices for different periods of time with osteoblastic cells; the

morphology of the cells seeded and cultured onto the scaffolds was examined by SEM (scanning electron microscope) and the cell viability and metabolic activity was evaluated using DNA and ALP assays.

5.2 MATERIALS AND METHODS

5.2.1 TEST MATERIALS

In this work, chitosan based porous structures were produced by means of combining a sol-gel process and the freeze-drying technique, as described previously [25, 33]. Briefly, chitosan (powder form) was dissolved in 0.2 M acetic acid solution at concentration of 4 wt% to obtain a homogeneous solution. Appropriate amounts of tetraethoxysilane (TEOS) and 0.5 M chloridric acid (HCl) solution were added to the chitosan solution under constant stirring for 24 and 72 hours. The method involves the hydrolysis and condensation of inorganic component (TEOS) in the chitosan matrix forming hydrogen bonding between the organic and inorganic phase. Then, the mixture was poured into Petri dish and allowed to dry at room temperature for several days. By its turn, the porous hybrids were obtained by transferring the mixture into a mould, freezing at -80 ^{o}C overnight, followed by freeze-drying for the period of 4 days to completely remove the solvent. The neutralization of porous hybrids was performed by soaking in a 0.1 M sodium hydroxide solution for 10 min and washing abundantly with distilled water until reaching the pH 7.0. Afterward, the porous structures are placed again in the freezing at -80 ^{o}C overnight, followed by freeze-drying for the period of 2 days. The scaffolds conditions tested and the correspondent sample designation is presented in theTable 5-1. All the samples/scaffolds obtained, corresponding to different formulations, were cut into cylinders of 0.6 cm (b) x 0.3 cm (h) x 0.25 cm (r) and sterilized by ethylene oxide prior to all the biological studies performed.

Table 5-1. Conditions of the several chitosan porous hybrid scaffolds

Sample	polymer (g)	TEOS:HCl (wt%)	$CaCl_2$ (g)	Reaction time (hours)
CT5	79.2	0.1:0.1	-	72
CT6	79.2	0.1:0.1	0.0788	72
CT7	79.2	0.1:0.01	-	72
CT8	40	0.1:0.1	-	24

5.2.2 CELL CULTURE STUDIES

A cell line of osteoblastic like cells-SaOS (human osteosarcoma) was selected for the culture studies as the developed 3D-chitosan scaffolds are aimed to be used in bone tissue regeneration applications.

The cell line (SaOS) was obtained from European Collection of Cell Cultures (ECC, UK) and was cultured in 75 cm^2 culture flasks, using Dulbecco's Modified Eagle's Medium-low glucose (DMEM-Sigma-Aldrich, Portugal), supplemented with 10 % foetal bovine serum (FBS, Biochrom AG, Germany), 1% antibiotic/antimycotic (Invitrogen, Portugal) and sodium bicarbonate (Sigma, Portugal) at 37 ^oC in a 5 % CO_2 incubator [41]. The culture medium was changed every 2 days and cells were grown until confluence, before starting the *in vitro* assays. Chitosan based scaffolds were seeded with cells and cultured for different periods of time. The cells adhesion and proliferation onto chitosan scaffolds developed using different formulations was assessed by scanning electron microscopy (SEM) and using the DNA and ALP assays as described bellow.

5.2.3 SEEDING AND CULTURING OSTEOBLASTIC-LIKE CELLS INTO 3D-CHITOSAN SCAFFOLDS

Osteoblasts cells were harvested before reaching confluence from 75 cm^2 culture flasks using trypsin-EDTA (Invitrogen, Portugal), re-suspended in 5 ml DMEM culture medium and centrifuged. Cell suspension was prepared in order to seed 5 x 10^5 cells per sample. The scaffolds were placed in 48 well non-adherents culture plates. For each different scaffold material was placed 6 samples and 300 µl of cell suspension was seeded onto the surface of each scaffold. The culture plate was transferred in a

5% CO_2 incubator for 3 hours at 37 ^oC. After this time, 1 ml of DMEM culture medium was added into each well-culture plate and the samples cultured for different periods of time (3, 7 and 14 days). The culture medium was changed every two days. At the end of each culturing period-scaffolds were washed with PBS and transferred to 15 ml tubes containing 1 ml of ultra-pure water and frozen at -80 ^oC before using for DNA and ALP analysis. The remaining scaffolds were further treated for SEM observations.

5.2.4 DNA QUANTIFICATION IN LYSED CELLS

The DNA content of each scaffold was measured using a Picogreen® dsDNA Quantitation Kit (Invitrogen/Molecular Probes) [49]. PicoGreen® dsDNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantify double-stranded DNA (dsDNA) in solution [50].

For the DNA quantification, the samples were defrosted using ultrasound bath for about 15 minutes. Afterwards, 28.7 µl of sample or standard + 71.3 µl of PicoGreen solution and 100 µl 1X TE (n = 4) were mixture using 96 culture plates (white opaque). The standard solutions were prepared in ultra pure water from 0.0 to 2.0 v/v. Then, the culture plates were incubated in the dark for 10 minutes. The fluorescence was read in the microplate reader (BIO-TEK – Synergy HT) at 485/20 nm for excitation and 528/20 nm for emission. DNA concentration was read from standard graph. The DNA quantification of each sample was calculated by correlation with the DNA of a known amount osteoblasts (SaOS) [49].

5.2.5 ALKALINE PHOSPHATASE (ALP) ASSAY

ALP is an enzyme secreted from osteoblasts. It is thought to promote crystal formation in matrix vesicles by removing nucleation inhibitors. It is inhibited by glucocorticoids and parathyroid hormone (PTH) [51].

In this work, the ALP activity was measured using a Sigma Diagnostic kit [49], where alkaline phosphatase activity from the scaffolds-cells samples was quantified by the specific conversion of *p*-nitrophenyl phosphate (*p*NPP) to *p*-nitrophenol (*p*NP). For the ALP quantification, the samples were prepared as describe for DNA assays, where

the samples were defrosted using ultrasound bath for about 15 minutes. Following from each tube/scaffold it was taken 20 µl of sample solution and added to a 96 well culture plate (*n* = 4). Then, 60 µl of substrate solution ALP (p-nitrophenyl phosphate - *p*NPP) was mixed. The 96-well plate was incubated at 37 ^{o}C for 45 minutes in the dark. After this period the reaction was stopped using 80 µl of stop solution and then the standard solutions were placed in the same 96-well plate (the standard solutions were prepared according to Table 5-2**Error! Reference source not found.**). The absorbance was determined at 405 nm using the microplate reader (BIO-TEK – Synergy HT) [42, 49]. Product concentration was read from standard graph. The results were expressed in *p*NP produced by sample/day of cell culture.

Table 5-2. Preparation of standard solutions - ALP assays

pNP Stock solution	Stop solution [µl]	Standard concentrations [µmol/ml]
0.0	160.0	0
0.8	159.2	5
1.6	158.4	10
2.4	157.6	15
3.2	156.8	20

5.2.6 SEM ANALYSIS OF THE CELL SEEDED SCAFFOLDS

At the end of each culturing period, the chitosan scaffolds-cells samples were rinsed twice with phosphate buffered saline solution (PBS, Sigma-Portugal) and subsequently dehydrated using an increased concentration series of ethanol solutions (25%, 30%, 50%, 70%, 80%, 90% and 100% - each 30 min). The samples were then dried at room temperature, and sputter coated with gold before observation in a scanning electron microscope (SEM, Leica Cambridge S360).

5.3 RESULTS AND DISCUSSION

5.3.1 CELLULAR ADHESION AND PROLIFERATION-SEM ANALYSIS

Chitosan based scaffolds were cell seeded with a SaOS cell line in order to evaluate the cell/material interaction with respect to cell adhesion and proliferation. The SEM

analysis performed after 3, 7 and 14 days of cell culture, revealed that the cell attachment and proliferation of the osteoblasts cells in contact with all scaffolds formulations surface was low. Only for the CT6 formulation a good response during all the culture time could be observed.

According to SEM micrographs (Figure 5-1), the best scaffold formulation was the CT6 as it was possible to observe cell growth, attachment and spread on its surface after only 3 days of culture. It is possible to detect cells colonizing the surface and inner regions (porous) of the scaffold. The rest of the formulations showed a very different behaviour, i.e., the samples exhibited lower cell adherence with cell cluster formation.

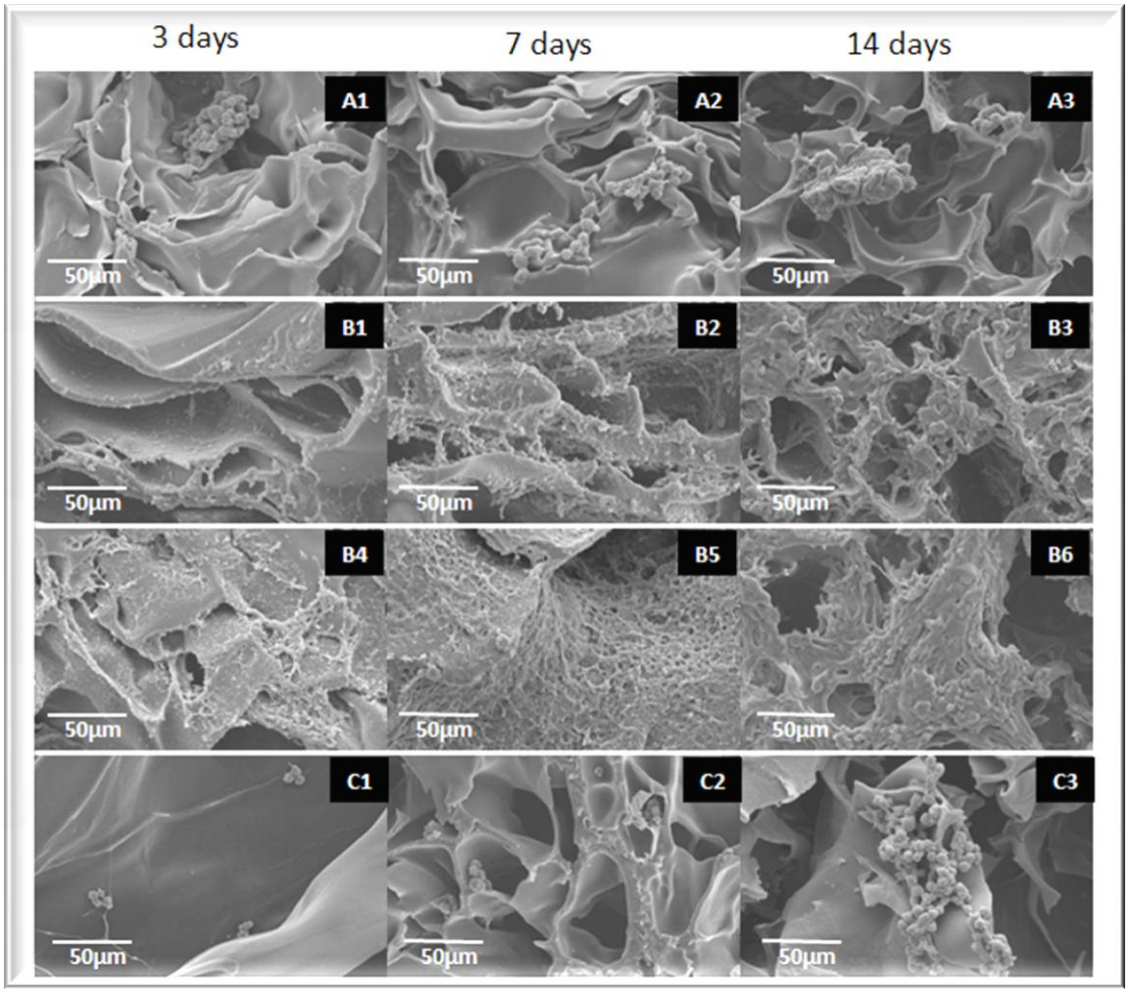

Figure 5-1. SEM micrographs showing SaOS osteoblasts cultured on chitosan scaffold after 3, 7 and 14 days of cell culture. A1 to A3) CT5 scaffold, B1 to B6) CT6 scaffold, C1 to C3) CT7 scaffold.

The different behaviour of the different formulations of chitosan scaffolds (non homogeneous cell distribution and lower cell attachment) can be explained by the monopolar basic nature of chitosan, which does not interact well with the bipolar extracellular matrix proteins present in the bovine serum proteins of the culture medium [52].

In the case of CT6 formulation, the higher cell growth could be explained by the content of $CaCl_2$ in the mixture with the polymer. Indeed, the osteoblasts cells have affinity with calcium for the formation, secretion and mineralization of extracellular bone [9, 51]. Additionally, the quantity of polymer (79.2 g) and reaction time (72 h) can affect cell proliferation. Also, the addition of calcium chloride provides Ca^{2+} ions that associated to presence of silanol groups (Si-OH) in these materials can induce the biomineralization [53].

Previous chitosan scaffolds studies suggested good cell proliferation and differentiation in porous structures based chitosan [29, 30, 35]. However, in such studies it was developed 3D-scaffolds using different chitosan combination such as chitosan/hydroxyapatite [30], chitosan/gelatin [29] and chitosan/chondroitin sulphate [54] using different techniques such as sintering and a freeze-drying technique [30], solid-liquid phase separation and subsequent sublimation [29] and interpolyelectrolyte complex/lyophilization method [54].

In this particular study, the low cell adhesion of osteoblasts generally observed in the SEM analysis on chitosan porous scaffolds suggests the need for further improvements regarding the scaffolds formulations, in order to promote a better cell adhesion in these materials, as well in the seeding protocol.

5.3.2 DNA ANALYSIS

Figure 5-2, shows the results obtained by DNA assay after different periods of culturing into chitosan based scaffolds correspondent to different formulations. The results demonstrated a low cell density after 3 days culturing.

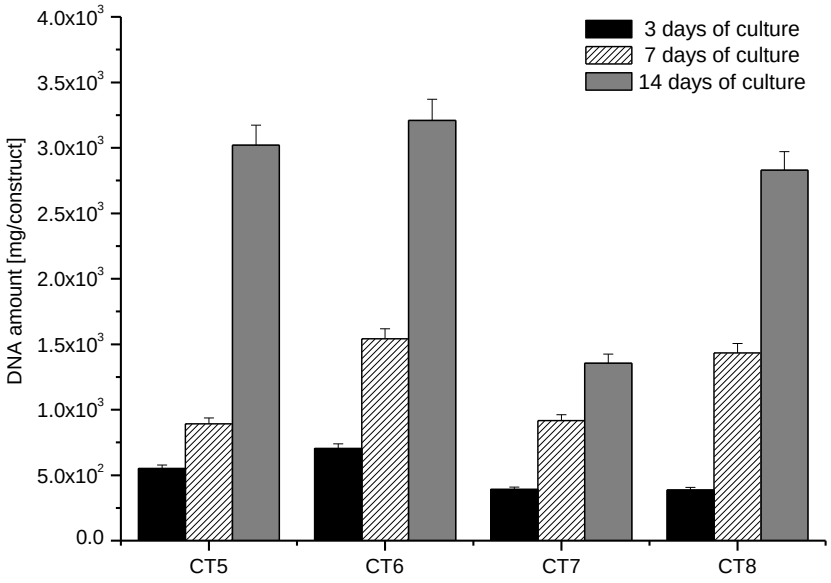

Figure 5-2. Number of cells on chitosan based scaffolds with different formulations after 3, 7 and 14 days of cell culture obtained in the DNA Quantification assay.

However, DNA values showed a significant and consistent enhancement after 7 and 14 days of culture for all formulations comparatively to 3 days culturing (Figure 5-2). In fact, the results obtained from the DNA assay showed the occurrence of cell growth and attachment in all the porous chitosan constructs. DNA results are not in agreement with the SEM observations which may be explained by the detachment of cells during the procedure for preparing samples for SEM observations.

Nevertheless, the number of cells present in the scaffolds after 7 and 14 days of culture are still low if compared to the amount of cells initially seeded which may be explained by a low cell-seeding efficiency due to the procedure used and/or to the nature of the surface of the chitosan scaffolds, that may not promote significant initial cell attachment, as it was demonstrated in previous chapter for chitosan based membranes without any surface treatment.

5.3.3 ALP ANALYSIS

The functional activity of the osteoblasts on the chitosan scaffolds was determined by quantifying the ALP activity. Figure 5-3, shows the ALP activity obtained after different culture periods of the chitosan based scaffolds with different formulations. This assay demonstrated the relations of enzymatic activity of osteoblasts cells with cells number reported in DNA quantification assay. It is clear that several formulations exhibited a constant and increasing enzymatic activity with increasing culture.

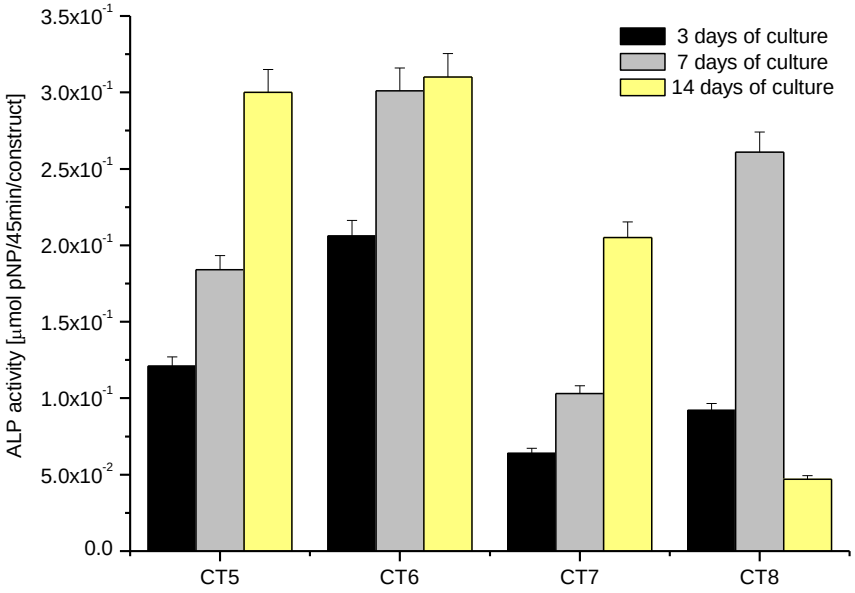

Figure 5-3. ALP activity of osteoblasts cells after 3, 7 and 14 days of cell culture on chitosan based scaffolds.

The increase of ALP activity with culture time demonstrates a consistent growth of cells and cell metabolic activity in the porous chitosan structure as reported in previous studies [29], where the existence of a ceramic phase, plays an important role. The data in Figure 5-3 is fully consistent with the DNA analysis results (Figure 5-2) strengthening the hypothesis that some of the formulations that were tested are able to support cell development within their structures.

5.4 CONCLUSIONS

The biological response of chitosan scaffolds cross-linked with TEOS, through a sol-gel process, was accessed. Except one of the formulations all the other formulations demonstrated low initial cell attachment and proliferation.

However, after 3 days of culture the cells start to proliferate and grow over the studied culture time (14 days). This suggests that, as demonstrated for chitosan membranes, the chitosan scaffolds may need to be submitted to a surface treatment (such as plasma treatment) to enhance cell attachment on the surface of these scaffolds. Additionally, it may also be required the optimization of the scaffolds formulations used, as clearly one of the formulations used resulted better than the remaining ones, in terms of initial cell attachment and growth.

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CHAPTER 6

6 GENERAL CONCLUSIONS

The present work had two major goals, the first was to study the biological response of chitosan based membranes and porous 3D-scaffolds, that are being studied for applications as supports for growing tissue substitutes, in special for the regeneration of skin and bone tissue, while the second aimed at the development of new systems for cell encapsulation based on two natural polymers, alginate and carrageenan, that can be used in different tissue regeneration or other cell based therapies.

The main conclusions of this thesis are presented according to the above mentioned objectives.

BIOCOMPATIBILITY OF CHITOSAN BASED MEMBRANES AND SCAFFOLDS AND MODIFICATION OF CELL ADHESION BY PLASMA SURFACE TREATMENTS

The MEM extraction and MTT test showed that the leachables released by all membranes formulations studied were not harmful to the cells. Additionally, in both assays, changes in cell morphology and cell metabolism were not evidenced.

The surface of the chitosan membranes modified by plasma treatment, using argon and nitrogen gas showed, in all the *in vitro* assays performed, enhanced cell attachment and proliferation, as compared to untreated membranes.

SEM observations of the cells seeded onto chitosan membranes showed that cell proliferation rates were similar in all treated surfaces using both plasma treatments, under different conditions. However, MTS showed some differences in cell proliferation, depending on the gas plasma type, demonstrating that nitrogen plasma produced better results as compared to argon plasma. The enhancement in cell proliferation observed in the membranes surface treated by plasma may be explained by the raise in the levels of oxygen and nitrogen obtained in the chitosan membranes

surface after these treatments. As a result, chitosan membranes treated by plasma surface modification demonstrated to be adequate for the cell adhesion and growth, demonstrating the high potential of chitosan membranes treated by plasma for future applications in the biomedical field, namely in skin regeneration.

Several formulations of chitosan based scaffolds were developed using methodologies that combine sol-gel process and freeze-drying techniques. In general, all formulations demonstrated low initial cell attachment and proliferation, except one of the formulations. This suggests that, as demonstrated for chitosan membranes, the chitosan scaffolds may need to be submitted to a surface treatment (such as plasma treatment) to enhance cell attachment on the surface of these scaffolds. Additionally, it may also be required the optimization of the scaffolds formulations used, as clearly one of the formulations used resulted better than the remaining ones, in terms of initial cell attachment and growth.

DEVELOPMENT OF NEW CELL ENCAPSULATION SYSTEMS BASED ON ALGINATE AND CARRAGEENAN

Microcapsules were developed using *iota* and *kappa* carrageenan as well as mixtures of *iota*-carrageenan with alginate. Chitosan was used to form the capsule's membranes. All capsules developed exhibited spherical shape, smooth surface and revealed good stability in PBS and in culture medium.

Iota-carrageenan constitutes soft and elastic gels, forming capsules with more permeability than *kappa*-carrageenan capsules, showing to be suitable for cell encapsulation.

The microcapsules based on mixture of sodium alginate and *iota*-carrageenan solutions showed that the mechanical properties of these capsules were mainly influenced by the alginate/carrageenan ratio used. The results of short-term stability evaluation suggested that capsules with high content of *iota*-carrageenan are more stable and their stability is not negatively affected by the citrate treatment.

Cell encapsulation experiments showed that these capsules maintained cell viability and did not form cell aggregates.

The results obtained suggest that this new polymeric system based on mixtures of alginate with *iota*-carrageenan produce adequate alternative systems for cell

encapsulation that may found several applications in tissue engineering or other cell based therapies. Nevertheless, these systems require future studies in order to demonstrate all their potential in biomdical applications.